Development of genetic crossing methods to identify genes associated with macrocyclic lactone resistance in the sheep nematode parasite, *Haemonchus contortus*.

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Abstract

There is a pressing need to develop strategies to reduce the emergence of macrocyclic lactone anthelmintic resistance in sheep flocks. Management practices aimed at maintaining anthelmintic susceptible nematodes *in refugia* while achieving a satisfactory level of production may prove to be useful. However, sensitive molecular tests are required to monitor the subtle effects of these practices on the frequency of resistance alleles within nematode populations. To-date, conventional studies of candidate genes coding for the known methods of action of macrocyclic lactone anthelmintics have produced a complex picture, highlighting the relevance of different approaches to the identification of resistance markers.

This thesis describes the development of a single nematode parent genetic crossing method and discusses its application to identify molecular markers for anthelmintic resistance. Parasitological and molecular verification of successful inbreeding of the MHco3 strain of *H. contortus* derived from the progeny of a genetic cross between single nematode parents is described. The single parent genetic crossing method has enabled the production of diverse inbred lines of the MHco3 *H. contortus* and may prove useful for genome assembly, or for the development of a genetic map. The study has afforded insights to the biology of *H. contortus* and effects of host immunity on nematode parasites. New information is presented concerning the period during which adult female nematodes continue to shed fertilised eggs after removal of males, the development of unfertilised *H. contortus* eggs, and the population genetics of mixed infections of two different strains of *H. contortus*.

Novel backcrossing experiments initially between a macrocyclic lactone resistant (MHco4 or MHco10) and a susceptible (MHco3) strain of *H. contortus* and then between ivermectin treated backcross generations and the parental susceptible strain are described. The resources provided by these experiments should enable comparative genomic analysis and conventional molecular biology to identify resistance genes derived from the parental resistant strains in fourth backcross generations that are the same as a parent ivermectin susceptible population, apart from the presence of alleles linked to anthelmintic resistance, derived from parent resistant strains.

Author’s declaration

I declare that this thesis contains my own original work, except where otherwise stated, and that it has not been submitted for any other degree or professional qualification.

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Abbreviations

A	adenine
AAD	amino-acetonitrile derivatives
ABC	adenosine triphosphate binding cassette
AMA	adult migration assay
AMOVA	analysis of molecular variance
APS	ammonium persulfate
BAC	bacterial artificial chromosome
C	cytosine
CAVR	Chiswick avermectin resistant
DDT	dichloro-diphenyl-trichloro-ethane
DMSO	dimethyl sulfoxide
DNA	deoxyriboucleic acid
dNTP	deoxynucleotide triphosphate
ED	egg death
EHA	egg hatch assay
EL_4	early fourth stage larva
epg	eggs per gram
F200Y	tyrosine to phenyalanine SNP at codon 200
FECRT	faecal egg count reduction test
FITC	fluorescein isothiocyanate
FWEC	faecal worm egg count
ISE	inbred SE
F_1	first filial
G	guanine
GABA	gamma amino-butyric acid
GluCl	glutamate gated chloride
H_e	expected heterozygosity
H_o	observed heterozygosities
ITS	internal transcribed spacer
IPTG	isopropyl β-D-1-thiogalactopyranoside
IVM	ivermectin
kb	kilo base pairs (1000 bp)
L_1	first stage larva

L_2	second stage larva
L_3	third stage larva
LB	Luria Bertani
LDT	larval development test
LFI	larval feeding inhibition
LFIA	larval feeding inhibition assay
LMI	larval migration inhibition
LMIA	larval migration inhibition assay
MPA	methyl progesterone acetate
mb	mega base pair
mRNA	messenger ribonucleic acid
NTS	non transcribed spacer
PCA	principal coordinates analysis
PCR	polymerase chain reaction
P-gp	P-glycoprotein
RFLP	restriction fragment length polymporhism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
T	thymine
TEMED	tetramethylethylenediamine
T_m	melting temperature
U	uracil
VMD	Veterinary Medicines Directorate
WAAVP	World Association for the Advancement of Veterinary Parasitology
WRS	White River strain
X-Gal	5-bromo-4-chloro-3-indoly-beta-D-galactopyranoside

Chapter 1: General introduction

1.1 The economic importance of parasitic gastroenteritis

Wherever livestock are farmed for economic production, the principal reason for keeping domestic ruminants is to convert primary forage or herbage crops into a marketable product. In most countries where sheep are farmed commercially, including the UK, the primary marketable product is meat. The efficiency of conversion of feed to meat is greater in lambs that achieve maximal growth rates than in ill thrifty lambs because all animals have a daily feed requirement for maintenance which must be met before growth can occur, irrespective of the time taken to reach slaughter weight. Furthermore, lambs which are slow to finish are more susceptible to compounding effects of production limiting diseases than rapidly growing animals which may leave the farm before the main risk period for these problems. The profitability of global sheep farming is therefore heavily influenced by the efficiency of feed conversion to meat.

The common causes of suboptimal weight gains in UK growing lamb flocks are: poor nutritional management; previous perinatal disease; parasitic gastroenteritis; cobalt deficiency; selenium deficiency; fascioliasis; and other specific infectious and management problems such as respiratory disease, lameness, sheep scab, coccidiosis and border disease (Sargison, 2008). Parasitic gastroenteritis is arguably the most important of these, often occurring concurrently with other problems (Sargison, 2004).

Globally, the major production limiting nematode parasite species are *Teladorsagia circumcincta* (formerly *Ostertagia circumcincta*) and *Haemonchus contortus* (Vlassoff and McKenna, 1994), although *Trichostrongylus vitrinus* and *Nematodirus battus* are also important in the UK, while *Trichostrongylus colubriformis* and various other

nematode species cause production loss elsewhere, and are sometimes relevant in the UK. While there is a general seasonal occurrence of parasitism by different nematodes, several species are usually present at the same time and in practice parasitic gastroenteritis is usually considered as a whole, rather than as diseases caused by specific nematode species.

Nematode parasites limit the productivity of susceptible animals because of their various feeding activities; evoking an immune response in their host (Greer, 2008), damaging the absorptive lining of the abomasum or intestine (Fox, 1977), removing nutrients from the ingesta, or in the case of *H. contortus*, feeding on blood (Jackson and Coop, 2007). The net effects of these activities are inefficient feed utilisation, fluid and electrolyte imbalances and anaemia. Clinical gastrointestinal parasitism of lambs is common, characterised by scour, poor body condition, open fleeces and a pot-bellied appearance, sometimes leading to the death of the affected animal, and is an important welfare concern (Sargison and others, 2006a). However, overall the greatest economic importance of nematode parasites is sub-clinical production loss arising from continuous low-level exposure to infective larvae (Coop, 1979). Daily exposure for 90 days to about *4000 T. circumcincta* or 2500 *T. vitrinus* infective larvae (L_3) can reduce liveweight gains of *ad libitum* fed sheep by up to 50 percent due to reductions in appetite and feed conversion efficiency (Coop and others, 1988). Continuous low level infection with *T. vitrinus* or *N. battus* can also result in impaired calcium and phosphorus retention and poor skeletal mineralisation (Coop and Field, 1983). Parasitic gastroenteritis also causes production loss because it can lead to reduced carcase values and due to the considerable cost incurred by its treatment and management. In 1988 sheep production losses to parasitic gastroenteritis in New Zealand, with a ewe population at that time of about 35 million, were estimated at $NZ275 million per annum (Brunsdon, 1988). These estimated losses were: $NZ27.7 million to lost meat production; $NZ149.2 million to lost wool production; $NZ93.2 million to poor ewe weight and subsequent poor fertility at mating; and $NZ5 million estimated cost of dag removal. Admittedly, the relative values of these losses have changed over recent years,

but New Zealand's total value of sheep products for export in 1996 was approximately $NZ2,500 million (Source: Annual Review of the New Zealand Sheep and Beef Industry, 1995-96). Thus the potential cost of gastrointestinal parasitism amounted to almost 15% of the country's sheep output. A comparable figure of £65 million per annum is commonly quoted in the farming press as the cost of parasitic gastroenteritis to UK sheep farmers.

1.2 *Haemonchus contortus* - a model parasitic nematode

1.2.1 The nematode parasite lifecycle of *H. contortus*

H. contortus is primarily a parasite of sheep and goats, but has a wider reservoir host range potentially including, cattle, some wild ruminants and even some small rodents. *H. contortus* is a diploid, obligate sexually reproducing species, with a direct heterogonic life cycle and a typical trichostrongylid pre-parasitic phase. Adult female nematodes within the abomasum or small intestine of their sheep host lay eggs, which are voided with faeces. Eggs hatch within the faeces, giving rise to first (L_1), then second stage larvae (L_2), which feed on faecal bacteria. L_2 then moult to infective third stage larvae (L_3). The L_3 are retained within the sheath of the L_2, providing protection from desiccation, but preventing them from feeding. L_3 find their way onto herbage, from where they are ingested by their sheep host. Ingested L_3 exsheath within the ruminoreticulum before passing into the abomasum and completing their development close to the gastric glands, moulting twice through fourth stage larvae (L_4) to adults. Late L_4 develop a tooth that enables them to pierce mucosal blood vessels and feed on blood. Adults then move freely over the abomasal mucosal surface. As is the case for most sheep nematode parasites, the pre-patent period between ingesting L_3 and significant fertilised egg shedding is between 17 and 18 days.

The environmental conditions that favour optimal *H. contortus* egg hatching and larval development are generally optimal at high humidity and temperatures above 24^{o}C. These temperature requirements for egg hatching and development and survival of the free living stages of *H. contortus* are higher than those of the other important parasitic nematode species affecting UK sheep. Given optimal conditions of temperature and moisture, egg hatching may occur within 24 hours of faecal shedding and larval development to infective L_3 may occur within as few as 5 days. The success and rate of egg hatching and larval development to L_3 is reduced at lower temperatures, where larval development may take several weeks, being minimal at temperatures below 10^{o}C. Eggs, L_1 and L_2 are easily killed by desiccation, but given adequate moisture, infective L_3 mostly survive on pasture for 10 to 12 weeks. Unlike the case of *T. circumcincta* or *T. vitrinus*, the survival of *H. contortus* L_3 is poor at temperatures below 10^{o}C and free living stages of the parasite are killed by frost. Thus, general environmental moisture and temperature have a major influence the epidemiology of haemonchosis through their effects on free living nematode populations on pasture. However, the temperature and moisture within specific microclimates such as sheep faeces can result in unpredictable larval survival during situations when general environmental conditions are unfavourable. For example, in southern parts of the UK, clinical outbreaks of haemonchosis are sometimes reported in autumn, when rainfall at the end of a period of drought enables the release of large numbers of larvae onto pasture from faeces, parts of which remained moist having been protected by a desiccated crust throughout the drought period.

1.2.2 The global importance of *H. contortus* as a sheep nematode parasite

Haemonchus contortus is the most important parasitic nematode of small ruminants worldwide due to its high pathogenicity as a blood feeding parasite. *H. contortus* late L_4 and adults in the abomasum feed on blood. Each adult nematode ingests about 0.05 ml of blood per day, so that sheep with burdens of just 500 adult *H. contortus* lose about 25

ml of blood daily (Taylor and others, 2007). Sheep suffering from acute haemonchosis may harbour burdens of 5,000 *H. contortus*. The blood feeding activity of adult *H. contortus* leads to haemorrhagic anaemia and protein loss, but in the absence of other gastrointestinal parasites, affected sheep do not scour (Sargison 2008). Digested blood is present in the faeces of affected sheep, due to seepage from the sites of feeding on the abomasal mucosal capillaries.

In tropical regions and in major sheep producing areas with hot climates and seasonal high rainfall such as parts of South Africa, Australia and South America, haemonchosis is a major constraint on sheep production and a serious animal welfare concern. Outbreaks of haemonchosis in these areas are often seen as a peracute or acute disease, characterised by profound anaemia, hypoproteinaemia, rapid weight loss and death involving large proportions of lamb and periparturient ewe flocks. The severity of disease outbreaks is determined partly by the level of infective larval challenge and partly by the consequence of inadequacy of available dietary protein nutrition on the host response to larval challenge (Roberts and Adams, 1990). In cases that follow sudden massive infective larval challenge, that can occur for example at the end of a drought period, peracute disease may be seen within 2 weeks of infection, characterised by severe haemorrhagic gastritis and the sudden death of previously healthy sheep. These peracute disease outbreaks illustrate the adaptability of *H. contortus*, to evolve strategies (such as developmental arrest of early fourth stage larvae (EL_4) (Blitz and Gibbs, 1972), or temporary reduction of egg laying to enable prolonged survival of adult female nematodes in the abomasa of their hosts (Frank Jackson, *personal communication*)) to enable its survival when conditions are unfavourable for free living stages, and to exploit favourable conditions rapidly (by the emergence of EL_4, or resumption of egg laying by female nematodes) when they return (Waller and others, 2004).

In warm and temperate climates, such as New Zealand and parts of Europe, including southern England, haemonchosis is more typically associated with ill thrift and

weakness. Anaemia and hypoproteinaemia are often unapparent until the later stages of the disease due to the adequacy of protein nutrition. Clinical signs of pallor of the ocular mucous membranes, submandibular oedema and ascites are only seen in individual, chronically infected animals. Unlike the situation in tropical and subtropical districts, where *H. contortus* infection frequently occurs in isolation from other nematode parasites, in temperate climates the parasite is usually present as a component of a mixed infection, causing parasitic gastroenteritis.

1.2.3 The relevance of *H. contortus* to UK sheep farming

The environmental conditions that are necessary for *H. contortus* egg hatching and larval development, and the fact that *H. contortus* eggs and larvae seldom survive over-winter on pasture in northern parts of the UK, have traditionally confined outbreaks of haemonchosis to warmer, southern districts (Rose, 1963). In most temperate regions of the world, including southern England, where over-winter survival on pasture of *H. contortus* eggs and developing larvae is limited, a situation is commonly described whereby the development of a proportion of the *H. contortus* L_3 acquired during late autumn becomes arrested as hypobiotic EL_4 within the abomasal glands of their sheep or goat hosts (Thomas and Waller, 1979; Waller and others, 2004). Development to adults is completed during the spring to coincide with the periparturient relaxation of immunity and presence of naïve lambs, thereby evading unfavourable conditions (Donaldson and others, 1998). Hypobiosis probably arises in response changing environmental conditions acting on developmental stages of the parasite, or on its sheep host (Brunsdon, 1973), although the precise stimuli have not been consistently demonstrated. Hypobiosis also occurs in warmer regions, but is not absolute, with wide variations in the proportion of EL_4 involved (Brunsdon, 1973; Uriarte and others, 2003), while in hot climates, hypobiosis is either not observed, or occurs as a parasite survival strategy during seasonal drought periods (Fritsche and others, 1993).

Traditionally, occasional cases of haemonchosis in northern England and Scotland, have mostly involved small numbers of sheep introduced from southern England. However, personal experience, supported by surveillance data shows a marked increase in outbreaks of haemonchosis in northern parts of the UK since about 2001 (for example, SAC Veterinary Science Division, 2001), in flocks with little or no recent history of introduction of sheep from southern England. While warmer spring and summer temperatures have probably afforded suitable new, more-northerly environments for egg hatching and larval development, it remains unlikely that significant over winter survival of the free-living stages *H. contortus* occurs on pastures in northern UK. It has, therefore, been suggested that the parasite has evolved to overcome this problem and exploit the opportunity presented by climate change by overwintering of virtually all infective larvae acquired during late autumn as hypobiotic fourth stage larvae (Sargison and others 2007b).

The first indication of haemonchosis in sheep flocks in southern Scotland has been sudden onset disease in ewes in early summer caused by the mass emergence of hypobiotic larvae. In some cases, sudden deaths have occurred due to severe haemorrhagic enteritis. Ill thrift in lambs during late summer has been reported as an inevitable consequence of pasture contamination by their dams (Sargison and others 2007b). Maturation of EL_4 has repeatedly been observed in sheep grazing on new pasture, but not on previously similarly managed animals from the same flocks grazing on older permanent pastures. This may provide a further example of the evolutionary adaptability of *H. contortus*, indicating that the maturation of hypobiotic parasites in the abomasum is somehow triggered by the provision of an environment that best favours egg hatching and larval development. Reports from Sweden (Waller and others, 2004) raise concern that *H. contortus* could become more important as a cause of acute disease and ill thrift in sheep throughout the UK, due to the parasite's evolutionary ability to adapt to new environments. Study of the population genetics of *H. contortus* in the UK is therefore pertinent (Gilleard, 2006).

1.2.4 Advantages of *H. contortus* as an experimental model parasite

The model free-living nematode, *Caenorhabditis elegans*, has been studied in great detail, providing a valuable source of anatomical, physiological and genetic information. The entire genome of *C. elegans* has been sequenced and annotated, providing a valuable resource for comparative genomic study. The experimental study of *C. elegans* is facilitated by its free living and hermaphroditic biology, while that of parasitic nematodes is hindered by their heterogonic life cycles and obligate bi-sexual reproduction. Unfortunately, these differences between free-living and parasitic nematodes limit the value of *C. elegans* for the study of traits expressed by parasitic nematodes, such as anthelmintic resistance.

H. contortus is the most amenable small ruminant parasitic nematode for experimental study, due to its large size, straightforward morphological identification, high fecundity and biotic potential, and relative ease of experimental infections. Anthelmintic resistance is widespread in *H. contortus* and it is the parasitic nematode species in which resistance has been most intensively studied to date.

The value of *H. contortus* as an experimental model parasitic nematode is further enhanced by the recent development of genomic resources. The Pathogen Sequencing Unit of the Wellcome Trust Sanger Institute, Cambridge, UK, is currently engaged in a sequencing and mapping project for the *H. contortus* genome. The aim of this project is to assemble a fully annotated genome sequence using a combination of shotgun sequencing and clone-by-clone bacterial artificial chromosome (BAC) and/or fosmid sequencing, to allow functional genomic approaches. The project is based on deoxyribonucleic acid (DNA) template produced from the inbred MHco3 (ISE) strain of *H. contortus* (Roos and others, 2004), that is maintained at the Moredun Research Institute (Frank Jackson, *personal communication*). Molecular biology support for the project has been provided by the Glasgow University Veterinary School (John Gilleard,

personal communication). At present about 600 megabases (mb) of shotgun sequence has been generated, representing about 6-fold genome coverage. This provides an extremely timely opportunity to apply genomic approaches to study of traits such as anthelmintic resistance in *H. contortus*.

The value of *H. contortus* for functional genomic experimentation is enhanced by its high biotic potential, and by the large size of the adult nematodes that allows for the preparation of large amounts of DNA from a single worm. The phylogenetic relationship between *H. contortus* and *C. elegans* is relatively close (Blaxter and others, 1998), lending itself to comparative genomic studies and eventually enabling the functional expression of *H. contortus* genes in *C. elegans* (Kwa and others, 1995; Gilleard, 2006).

The epidemiology of *H. contortus* in temperate regions follows an epidemic pattern, with rapid population expansions during the summer months, followed by bottlenecks caused by poor survival of free-living stages on pasture over winter. The consequent low level of gene flow and frequent population crashes lead to genetic drift (whereas *T. circumcincta* has a more stable population structure and lower genetic diversity (John Gilleard, *personal communication*)). Hence, genetic diversity both within (Troell and others, 2006) and between (Otsen and others, 2000b; Redman and others, 2008) populations of *H. contortus* is high, making the parasite amenable to study of population genetics and analysis of genetic crossing experiments. Furthermore, the high genetic diversity of *H. contortus* (Redman and others, 2008), coupled with its high fecundity and relatively short generation interval when conditions are ideal, increases the likelihood that a high frequency of resistance alleles will present in a population, underpinning its value as a model parasitic nematode for the study of anthelmintic resistance.

1.3 The essential need for effective anthelmintics for the control of nematode parasites

1.3.1 The principles of nematode parasite control in sheep

Heavily contaminated pastures may harbour more than 1,000 infective L_3/kg herbage, which can result in daily intakes of 5,000 L_3 per susceptible lamb. Under these conditions, while frequent treatment with a conventional, short-acting anthelmintic may improve animal performance for a few days, production losses are inevitable. While lambs that are exposed to a high level of infective larval challenge can achieve satisfactory growth rates subsequent to the onset of acquired immunity, their cumulative weight gains never match those of lambs which are only exposed to a small challenge. The underlying principle of nematode control in finishing lambs is, therefore, to limit their exposure to infective L_3 on pasture (Coop and others, 1982). Very low levels of infective larval challenge have negligible effects on productivity, while enabling the development of immunity, which may be important in store lambs or future replacement breeding stock. Sustainable control programmes in individual flocks are based on the commonsense application of knowledge of the farming system and of the relationship between pasture contamination, the availability of infective larvae on pasture, the build up of infection in sheep and the regulatory effects of acquired immunity (Sargison, 2008).

Pasture larval contamination (predominantly with *T. circumcincta* in Scotland) in spring arises both from over-wintered infective L_3 on pasture and from nematode eggs shed by recently-lambed ewes (Wilson and others, 2009). The egg output of lactating ewes, referred to as the periparturient rise, derives from nematodes which overwintered within the ewes, and from completion of the lifecycle of overwintered L_3 ingested with pasture after lambing (Gibson, 1973). The relative importance of these sources of pasture larval contamination differs from year to year with different winter weather conditions and sheep grazing management, varies between different regions of the UK, and depends on

the nematode genera involved. When ingested by naive lambs, these infective larvae give rise to adult nematodes, which accumulate over the summer months and contribute to subsequent pasture larval contamination, leading to disease. Exposure to infective L_3 on pasture can be minimised by: finishing lambs quickly before pasture L_3 burdens become production limiting (this strategy has positive knock-on effects, with lower overwinter larval survival and challenge during the subsequent spring); grazing susceptible sheep only on 'safe' pasture; or use of anthelmintics to suppress pasture larval contamination.

Pasture management to produce 'safe' grazing needs to be rigorous and few commercial sheep farms are able to provide sufficient safe pasture for the purpose of nematode parasite control in all susceptible sheep, without compromising the efficiency of crop or cattle production. Worldwide, most nematode control regimes therefore rely to different extents on the use of anthelmintics (Barger 1997).

1.3.2 Anthelmintic drugs

All of the broad spectrum anthelmintic drugs that are licensed at the time of writing this thesis for the control of nematode parasites in sheep belong to one of only three groups based on their mechanisms of action. The modern, currently-licensed, broad spectrum anthelmintics are grouped as: tubulin binding drugs (benzimidazoles and pro-benzimidazoles); ganglion blocking agents (imidazothiazoles and tetrahydropyrimidines); and macrocyclic lactones (avermectins and milbemycin). In addition, the salicylanalide derivative, closantel, has a narrow spectrum of activity against *H. contortus*.

Tubulin binding drugs bind to tubulin proteins in the cytoplasm of the intestinal cells of nematodes, preventing polymerisation of tubulin to form microtubules (Borgers and others, 1975; Lacey, 1988). Microtubules form the structural basis of many cellular

activities, including motility, secretion, co-ordination and glucose transport, thus susceptible nematodes die of starvation. Benzimidazole drugs also inhibit egg hatching (Southcott, 1963).

Ganglion blocking agents act in different ways as cholinergic agonists and cell depolarisers at nematode nerve ganglia, causing rapid onset sustained muscle contraction and reversible spastic paralysis (Coles and others, 1974). Paralysed nematodes are dislodged into the intestinal lumen. These drugs are not ovicidal.

The mechanisms of action of the macrocyclic lactone drugs involve an increase in membrane permeability to chloride ions (Turner and Schaeffer, 1989), which is time dependent (Gill and others, 1991). Their known effects on nematode parasites include reduced pharyngeal pumping, paralysis of body muscles and effects on the uterus, leading to failure to feed, move or lay eggs, respectively. The main drug targets are considered to be glutamate- and gamma-aminobutyric acid- (GABA) gated chloride channels, while non-specific targets of action such as the P-glycoproteins (P-gp) involved with trans-membrane drug efflux pumps may also prove to be important (Blackhall and others, 2008).

In the absence of anthelmintic resistance, these modern anthelmintic drugs, when given at the recommended dose rates, have a wide safety margin to both sheep and operator and are highly effective at removing most stages of nematode parasites from their host. Some drugs, in particular mebendazole, fenbendazole and levamisole are not always effective against EL_4 of the abomasal nematode parasites *T. circumcincta* and *H. contortus* (McKenna, 1974; Lancaster and Hong, 1977; Andrews, 2000), which may occasionally be of practical significance when ewes or lambs are treated during late autumn or winter (Sargison and others, 2007b).

To date, all new antiparasitic drugs have been developed through the random screening of synthetic or fermentive chemicals, such as botanical and industrial compounds (Geary

and others, 2004). These approaches have elucidated potential anthelmintic drug classes, such as the paraherquamides, cyclic depsipeptides and amino-acetonitrile derivatives (AADs). This process is both slow and expensive and no new class of anthelmintic drug for use in production animals has reached the market during the past 25 years. Advice on the responsible use of anthelmintic drugs is therefore based on the principle that this situation will continue. However, there is now a realistic probability that the development of the AADs will result in a marketable product (monepantel) within the foreseeable future (Kaminsky and others, 2008).

1.3.3 Alternative methods of nematode parasite control

Good nutrition and general disease management are prerequisites for effective nematode parasite control (Coop and Kyriazakis, 1999). Control of parasitic gastroenteritis in intensively managed sheep flocks will always rely on the strategic use of anthelmintics (Hennessy 1993b). However, currently unproven alternative strategies, such as breeding sheep for resistance to nematode parasites, the development of antiparasitic vaccines, manipulation of protein nutrition, grazing of condensed tannin-rich herbage species, or the use of nematophagous fungi may prove to be useful and important adjuncts to control of gastrointestinal parasitism in the face of emerging anthelmintic resistance problems, or in organic production systems.

Genetic selection for both host resistance and reduced susceptibility to nematode parasites could potentially reduce reliance on anthelmintic prophylaxis (Bisset and others, 1991; McEwan and others, 1997; Gray, 1997). Such lines of Romney, Perendale and Coopworth sheep have been selected in New Zealand since the late 1970s. However, the results of these earlier studies showed unfavourable side effects in the selected low faecal worm egg count (FWEC) lines when they were grazed alongside unselected sheep, including slower growth, poorer wool production and increased dags. These findings are consistent with observations that experimentally immune-suppressed

sheep develop greater nematode burdens than healthy sheep exposed to the same infective nematode larval challenge, but achieve higher growth rates; explained by the protein requirements of immunity and the fact that much of the pathology caused by nematode parasites arises due to the host's immune response (Greer and others, 2005a). Loose faecal consistency leading to perineal soiling may be due to the effects of mast cell inflammatory mediators in immune, resistant sheep when challenged with infective nematode larvae (Larsen and others, 1994; Williams and others, 2008). These problems may be surmountable and genetic selection using simple phenotypic markers, such as individual FWECs may prove to be a responsible strategy for some large UK sheep flocks. However, in the absence of reliable genetic markers that can be measured using a simple one-off blood test, selection for resistance to nematode parasites while minimising adverse consequences will be very slow. The application of selection for host resistance to nematode parasites relies on naturally acquired immunity, which is abrogated by nutritional and reproductive stress. Furthermore, the strategy may prove to be of little benefit where lambs are finished quickly, before the onset of acquired immunity.

The development of antiparasitic vaccines affords an obvious potential nematode control strategy (Munn, 1993; Smith, 1993; Emery, 1996). Various experimental methods have been tested, such as the oral administration of crude nematode homogenates, infection by abnormal routes and the use of irradiated attenuated larvae. The latter strategy affords effective protection against lungworm in cattle, but to date has been unsuccessful against gastrointestinal nematode parasites. Current research is focused on the understanding and potential manipulation of the host's immune mechanism (Smith, 2006) and the identification of target antigens on or secreted by the nematode (Knox and others, 2001). Some experimental success in terms of raised circulating antibody levels, reduced nematode egg output and reduced worm numbers has been achieved using crude vaccines against *H. contortus*, whose blood feeding activity means that molecules on the surface of its gut are appropriate vaccine targets. However, the development of recombinant sub-unit vaccines remains elusive. Furthermore, while single nematode

species vaccines may have a role in some countries, they are unlikely to be useful in the UK. Vaccination is, therefore, unlikely to provide a useful method of gastriontestinal nematode parasite control in the foreseeable future.

Biological control, aimed at free living sheep gastrointestinal nematode larval stages has been attempted using predatory nematophagous fungi, such as *Duddingtonia flagrans*. This strategy involves feeding fungal spores to sheep, faecal excretion of fungal spores alongside nematode eggs, and subsequent fungal development in the faeces, where their hyphae trap, kill and digest nematode larvae (Hashmi and Connon, 1989; Waller and Faedo, 1993; Grønvold and others, 1996). Efficient nematode trapping has been demonstrated under laboratory conditions, but the success rate in the field has been poor, associated with effects of temperature, diet, season, faecal consistency and larval density. Fungal spores survive passage through the sheep gut, but not long-term residence there, and an effective method of continuously delivering fungal spores to grazing sheep has not been found. Furthermore, any successful system would probably not be licensable in the UK due to environmental and residue considerations. It is therefore unlikely that nematophagous fungi will provide an alternative method of gastriontestinal nematode parasite control for use in UK sheep flocks.

Forage crops such as sulla, chicory, sainfoin and lotus have some bioactivity against gastrointestinal nematode parasites (Niezen, 1995; Waller and Thamsborg, 2004). These effects may be directly mediated by plant secondary metabolites such as condensed tannins, flavanols, or cysteine proteases, or indirectly mediated through enhanced host immunity resulting from optimised rumen degradable protein nutrition (Athanasiadou and others, 2005). Furthermore, the vertical migration and consequent availability of nematode larvae may be reduced on some alternative forage crops (Silangwa and Todd, 1964). While these bioactive forages may prove to be useful adjuncts to nematode parasite control in some countries, they do not withstand heavy UK grazing pressure. Furthermore, their bioactivity is not specific, potentially leading to toxic and anti-nutritional effects on grazing sheep as well as their nematode parasites.

1.4 Problems with anthelmintic control of nematode parasites

In the past, anthelmintic control of parasitic gastroenteritis was relatively straightforward for most sheep farmers. However in recent years scour, ill thrift and lamb deaths due to parasitic gastroenteritis have become common in the UK, often despite adherence to the basic nematode control principles. Several reasons have been identified for these problems including: exceptional over winter survival of infective nematode larvae on pasture associated with the effect of warmer autumn and winter weather on grazing management and larval survival (Sargison and others, 2002; Sargison and others, 2003); failure to suppress the post-lambing rise in worm egg output of lactating ewes grazing heavily contaminated pastures (Wilson and others, 2008), changes in the seasonal pattern of nematode parasitism (Van Dijk and Morgan, 2008); and anthelmintic resistance. These problems are frequently compounded by unsound advice about nematode control, constraints associated with reduced farm manpower and increasing flock sizes and inadequate handling facilities. Timely management to avoid production loss due to these problems depends on knowledge of the situation on each individual farm, based on the accurate diagnosis of previous disease outbreaks, strategic monitoring of FWECs to establish the pattern of nematode parasitism and assessment of the risks.

1.4.1 The threat to sustainable sheep production posed by anthelmintic resistance

1.4.1.1 Working definition of anthelmintic resistance

Anthelmintic resistance refers to a status where there is a greater frequency of individuals within a helminth population able to tolerate the recommended therapeutic dose of an anthelmintic drug than there is in a normal population of the same helminth species (Prichard and others, 1980). Anthelmintic resistance is a result of selection acting on genetic variation within the helminth population and is heritable (Martin, 1987).

1.4.1.2 The economic importance of anthelmintic resistance

Most sheep farmers rely on the use of effective anthelmintic drugs for the control of gastrointestinal parasitism. However, on some farms the usefulness of these drugs is reduced because of the presence of anthelmintic resistant nematodes. The first indications of the presence of anthelmintic resistance are often the failure of lambs to reach finished weights by late autumn, scouring and even deaths due to parasitic gastroenteritis, despite preventive anthelmintic treatments (Hughes and Seifert, 1983; Pomroy and others, 1985; West and Probert, 1989; Borgsteede and others, 1991; Orpin, 1991: Sargison and others, 2001). However, anthelmintic resistance can result in clinically in-apparent, sub-optimal growth rates for some time before these overt signs of disease are seen (Barger, 1995, Wilson and Sargison, 2007). The economic impact of anthelmintic resistance is further complicated by the fact that provided that nutrition is good, healthy and productive sheep can be maintained on a farm with a minimum anthelmintic efficacy of 80% (Barnes and others, 1995), although this figure is not sustainable, as the resistant nematodes will make ever more significant contributions to following generations. Furthermore, once the existence of anthelmintic resistance has been established, there are various managemental strategies that can be employed to mitigate against its potential economic effect.

The emergence of anthelmintic resistance is an inevitable consequence of good nematode control (Van Wyk, 2002), and not a result of bad farming practice (Kaplan, 2004). To date there is no evidence to show that anthelmintic resistant nematodes are any more pathogenic than non-resistant nematodes, so resistance itself is not production limiting. (An opinion has been expressed that anthelmintic resistant nematodes may be more pathogenic than susceptible nematodes (Georg Von Samson-Himmelstjerna, *examiner's comment*, citing Gerald Coles), based on observations that ivermectin resistant *Cooperia* spp. in cattle can cause scour, while ivermectin susceptible *Cooperia* spp. are generally considered to be less pathogenic (Vermunt and others, 1996). However, these observations may simply reflect an absence of modulating effects of

concurrent *Ostertagia ostertagi* in the abomasum on numbers or feeding activities of *Cooperia* spp. in the proximal small intestine of infected cattle (West and others, 1994). This situation could arise where the *O. ostertagi* are susceptible to ivermectin while the *Cooperia* spp. are resistant.) However, effective nematode parasite control in the face of multiple anthelmintic resistance is complicated, usually involving fundamental and sometimes expensive changes to sheep production systems. Nevertheless, in most cases, gastrointestinal nematodes can still be adequately controlled and it is important to keep the existence of multiple anthelmintic resistance in individual flocks in perspective (Larsen and others, 2006).

New Zealand and UK sheep farmers annually invest approximately $NZ30 million (Vlassoff and McKenna, 1994) and £10 million (regularly cited in the farming press) respectively on the cost of anthelmintics in order to minimize production loss due to parasitic gastroenteritis. Without these drugs, production losses due to parasitic gastroenteritis could be double the current estimates of $NZ275 million and £65 million respectively. Clearly, therefore, even where the effect of anthelmintic resistance on production is small, there will be a significant associated economic loss (Macchi and others, 1999). The continued use of anthelmintics to which resistance has developed has been conservatively estimated to reduce the liveweight gains of New Zealand lambs by 0.5 to 2.0 kg over the period between weaning and finishing (Mulvaney, 1995; Macchi and others, 2001). Anthelmintic resistance is now considered to threaten the profitability of the entire Australian sheep industry (Besier and Love, 2003), in particular where farming incomes are already under pressure. The potential emergence in the UK of a high prevalence of sheep parasites which are resistant to all of the three classes of broad spectrum anthelmintics presents the single most important sheep disease threat to economic and nationally sustainable lamb production (Sargison and others, 2005).

1.4.1.3 Early history of anthelmintic resistance

Anthelmintic resistance has been reported in nematode parasites of sheep, cattle (Jackson and others, 1987), pigs (Roepstorff and others, 1987), horses (Britt and Clarkson, 1988; King and others, 1990), and a number of exotic animal species, but remains of greatest importance in small ruminants. In general, anthelmintic resistance first appeared in the parasite genera which are hard to kill by the particular anthelmintic, for example in sheep, *H. contortus* resistance to benzimidazoles, in cattle, *Cooperia* spp. resistance to ivermectin (Vermunt and others, 1996; Coles and others, 1998a) and in horses, *Parascaris equorum* resistance to ivermectin (Stoneham and Coles, 2006).

1.4.1.3.1 Phenothiazine

The first report of nematode resistance to anthelmintics originated from the United States of America (Drudge and others, 1957), involving a strain of *H. contortus* resistant to the ungrouped drug, phenothiazine, only 20 years after the introduction of the drug (Gordon, 1945).

1.4.1.3.2 Benzimidazoles

The first benzimidazole anthelmintic, thiabendazole, was introduced during the early 1960s (Brown and others, 1961), and resistance in *H. contortus* was identified in New South Wales, Australia, after only 5 years (Drudge and others, 1964; Smeal and others, 1968). Almost all of the subsequent reports of benzimidazole resistance during the 1970s were from New South Wales and Victoria, Australia, mostly originating from research farms in Australia, where the frequency and period of anthelmintic drenching had been high. Resistant strains of *Trichostrongylus* spp. (Hotson and others, 1970), *H. contortus* (Le Jambre and others, 1976) and *T. circumcincta* (Le Jambre and others, 1977) were identified. Side resistance between different benzimidazole drugs was soon identified as the normal status (Hall and others, 1978). A series of farm surveys during the early 1980s identified the high prevalence of benzimidazole resistance on between 30% and 60% of commercial properties in the major sheep farming areas of Victoria, New South Wales and Western Australia (Webb and others, 1979; Cameron and others,

1984; Edwards and others, 1986; Webb and Ottaway, 1986). In Australia anthelmintic resistance is now the normal expectation, rather than the exception, with benzimidazole resistance present on approximately 85% of sheep properties (Overend and others 1994; Rolfe 1997).

Benzimidazole resistance has subsequently been identified, in New Zealand (Vlassoff and Kettle, 1980; Kemp and Smith 1982; McKenna 1989), throughout mainland Europe (Borgsteede 1986; Kerboeuf and others 1988; Borgsteede and others 1991), South America (Echevarria and Trindade 1989), Africa (Schalkwyk and Schroder 1989; Maingi 1991) and Asia (Van Aken and others 1989).

1.4.1.3.3 Ganglion blocking agents

Nematode resistance to the ganglion blocking anthelmintics, levamisole and morantel was first reported during the mid-1970s (Le Jambre and others, 1976) with side resistance between the two drugs (Le Jambre and Martin, 1979; Whitlock and others, 1980). The problem of multiple resistance (Prichard and others, 1980) was first reported in 1979, in New South Wales (Hall and others, 1979), involving a strain of *T. circumcincta*, resistant to both thiabendazole and levamisole. Levamisole resistance is now believed to be present on more than 65% of sheep farms in Australia, and benzimidazole-levamisole combination resistance on about 60% (Overend and others 1994; Rolfe 1997). It is estimated that only 9% of Australian sheep farms are free from anthelmintic resistance (Overend and others 1994). In each of these early Australian reports of resistance to the ganglion blocking agents, co-resistance to thiabendazole was also identified (Prichard and others, 1980).

1.4.1.3.4 Macrocyclic lactone anthelmintics

Ivermectin resistance was first reported in *H. contortus* in the Cape Province of South Africa in 1986 (Anonymous, 1986; Carmichael and others, 1987), just three years after its introduction to the market. The affected sheep had been grazed intensively and treated with ivermectin every 3 to 5 weeks over an 18 to 24 month period. Ivermectin

resistance was reported in a further five South African field strains of *H. contortus* during the following year (Van Wyk and Malan, 1988). Benzimidazole resistance was already present in all of these strains and in two flocks ivermectin resistance had emerged rapidly after only 3 flock treatments, despite its annual rotation with chemically unrelated anthelmintic drugs. Another farm in the White River region, in the Lowveld of the Transvaal (the origin of the benzimidazole and ivermectin resistant Hco4 White River strain), provided a detailed nematode control history, showing that ivermectin resistance had arisen after only 11 flock treatments, which had been well interspersed with the use of other anthelmintics, raising concern over the possibility of cross resistance between ivermectin and another anthelmintic group (Van Wyk and others, 1989). The only method of nematode parasite control used for most flocks grazed on irrigated South African pastures involved suppressive anthelmintic treatments at intervals of 3 weeks or less. *H. contortus* has a minimum prepatent period of about 18 days and oral ivermectin has a residual action of a few days. Thus this routine practice of administering ivermectin at intervals of 21 days would have allowed ivermectin susceptible *H. contortus* no opportunity to produce ova and thus propagate susceptible individuals in the population. Other South American reports of ivermectin resistance in *H. contortus* followed (Souza and others, 1993). In each case, pre-existing benzimidazole resistance was demonstrated. It was estimated in 1997 that *H. contortus* resistant to 3 or more anthelmintic groups was present in about 40% of South African sheep flocks (Van Wyk and others, 1997a), this high prevalence having arisen within 10 years of the first report of a benzimidazole and ivermectin resistant strain of *H. contortus* (Van Wyk and Malan, 1988).

Ivermectin resistance in *H. contortus* was not reported in Australia until 1993 (Le Jambre, 1993). The diagnosis of ivermectin resistance was only made following the serendipitous contamination of a laboratory strain of *T. colubriformis* and morphologically marked, ivermectin susceptible *H. contortus* with an extraneous wild strain of *H. contortus*. Three possible sources of iveremctin resistance were considered: firstly that resistance was selected from the laboratory strain following previous

ivermectin treatments; secondly the unlikely possibility that spontaneous mutation to ivermectin resistance had occurred, as has been shown the model nematode organism, *C. elegans*; and thirdly that quarantine procedures involving dosing sheep with ivermectin and holding them on yards for 16 hours before release to paddocks had failed to prevent the introduction of ivermectin resistance with purchased animals. This ivermectin resistant strain of *H. contortus* was subsequently characterised as the Chiswick avermectin resistant (Hco10) strain. Ivermectin resistant nematode parasites are now widespread throughout Australia (Waller, 2003).

The first report of ivermectin resistant *T. circumcincta* in Australia was in 1994 (Swan and others, 1994). Ivermectin had only been used on 3 or 4 occasions each year and resistance was considered to have developed primarily due to the selection pressure imposed on nematode populations by standard, recommended (Anderson, 1973) anthelmintic treatment regimes at the beginning of the hot and dry summer period (Anderson, 1983). Treatments at these ecologically critical times, when few infective larvae would have survived on the pasture, would have enabled those resistant nematode parasites remaining in the sheep to make up a greater proportion of the overall population following egg development to infective larvae when autumn weather conditions permitted. Four further cases of ivermectin resistance were reported in *T. circumcincta* in Western Australia in 1996, also identifying the practice of anthelmintic treatments at the start of the hot dry summer as being the most likely risk factor (Besier, 1996). In 2001, the prevalence of ivermectin resistant *T. circumcincta* in Western Australia was estimated to exceed 38% (Palmer and others, 2001), this situation having arisen within 7 years of the first report.

The avermectin drugs and milbemycin have common methods of action and side resistance exists between the different macrocyclic lactone drugs (Watson and others, 1996).

1.4.1.4 Reversion to anthelmintic susceptibility

Whether or not reversion to susceptibility occurs is relevant with regards to determining management strategies to reduce the risk of anthelmintic resistance and to the management of nematode parasitism using anthelmintics once resistance has emerged to a particular class of anthelmintic. Reversion could potentially occur if resistant nematodes are disadvantaged, or less fit than susceptible nematodes, or if counter selection for susceptibility occurs. Some early reports suggested that reversion of anthelmintic resistant parasites to susceptibility might occur in *T. circumcincta* (for example, Martin, 1987) and *T. colubriformis* (for example, Waller and others, 1988; Waller and others, 1989). Subsequent studies have shown that while some degree of reversion to susceptibility may been seen when benzimidazole anthelmintics are first used against resistant *T. circumcincta* after a period of several years, resistance rapidly re-emergences to former levels within one or two treatments (for example, Hall and others, 1982; Jackson and Coop, 2000). In the case of *H. contortus*, no reversion to benzimidazole susceptibility has been reported following several years' reliance on another anthelmintic class (Herd and others, 1984; Borgsteede and Duyn, 1989). The possibility has been considered that resistant genotypes of *H. contortus* may be fitter than susceptible genotypes, thus influencing the potential for reversion. There are no published reports of significant reversion to susceptibility for the imidazothiazole or macrocyclic lactone classes of anthelmintics in sheep, but field data would suggest that it does not occur (Le Jambre and others, 1982). In goats, reversion to susceptibility to ivermectin did not occur following a 5 year period when macrocyclic anthelmintics were not used (Pomroy and others 1998).

1.4.1.5 Anthelmintic resistance sheep nematodes in the UK

Anthelmintic resistance was first reported on a sheep farm in England in 1982, with the identification of a benzimidazole resistant strain of *T. circumcincta* (Britt, 1982). Two more strains of benzimidazole resistant *T. circumcincta* were reported in southern England during the following year (Cawthorne and Whitehead, 1983). A survey in 1984 established the presence of thiabendazole resistant *H. contortus* populations on 7 out of

52 commercial farms in the south of England (Cawthorne and Cheong, 1984). A subsequent survey indicated that benzimidazole resistance was widespread in parts of England (Coles and others, 1991). Benzimidazole resistance was first reported in Scottish sheep in 1991 (Mitchell and others, 1991). A survey of sheep farmers in the south east of Scotland conducted in 2000 indicated that benzimidazole resistance in *T. circumcincta* may be present in as many as 81 percent of lowground flocks, 56 percent of upland flocks and 45 percent of hill flocks (Bartley and others 2001). Benzimidazole resistant *H. contortus, T. circumcincta*. and *Trichostrongylus* spp. in goats were reported in 1989 (Scott and others, 1989), 1991 (Jackson and others, 1991) and 1992 (Jackson and others, 1992a) respectively.

In 1996, levamisole resistance was identified in *T. circumcincta* in a survey of sheep farms in southern England (Hong and others, 1996; Coles and Simkins, 1996). However, there have been few further reports in UK sheep (Coles and others, 1998b) of resistance to imidazothiazole anthelmintics alone.

Populations of *T. circumcincta* resistant to all three anthelmintic groups (benzimidazoles, imidazothiazoles and avermectins) were identified in a goat flock in the south of Scotland during the 1980s (Jackson and others, 1992b). The first report of ivermectin resistance in sheep was in 2001, in a population of *T. circumcincta* in the south-east of Scotland that was also resistant to benzimidazole and imidazothiazole anthelmintics (Sargison and others, 2001). Several further populations of *T. circumcincta* resistant to benzimidazole, and macrocyclic lactone anthelmintics have been reported in the south-east of Scotland (Bartley and others, 2004; Sargison and others, 2004; Bartley and others 2006; Wilson and Sargison, 2007) and south of England (Cheng and others, 2003). A survey of sheep farms in the south-east of Scotland in 2004 showed evidence of ivermectin resistance in 30% of flocks (Bartley and others, 2006). There have also been several unpublished reports of multiple anthelmintic resistance, mostly in the southeast of Scotland, northeast of England and Northern Ireland. In each of these cases, the diagnosis of multiple anthelmintic resistance was first made when the

cause of poor lamb growth rates was investigated, typically after terminal sire cross lambs had failed to reach slaughter weights by 6 months-old. Future control of parasitic gastroenteritis will prove difficult in these flocks and economic sheep production may not be sustainable (Sargison and others, 2005).

Diagnoses of multiple anthelmintic resistance have been reported in less than 0.01% of the UK sheep flock. However, this figure may not provide a true indication of the national prevalence of the problem. Multiple anthelmintic resistance was reported in a pedigree Suffolk flock in 2004 (Sargison and others, 2004). The nematode control practices adopted by this particular flock, which might select for anthelmintic resistance, were similar to those used in most UK terminal sire flocks, from which sheep have been disseminated to commercial flocks throughout the country. *T. circumcincta* carrying genes conferring multiple anthelmintic resistance are therefore probably present in commercial sheep flocks throughout the UK, but remain unrecognised because they are present at a low frequency that cannot be detected using current insensitive diagnostic tests, and because few UK sheep farmers routinely check the efficacy of the anthelmintic which they are using (Coles, 1997; Sargison and Scott, 2003). Sheep farmers in the south-east of Scotland, where multiple anthelmintic resistance was first reported, may have been more proactive in identifying the cause of poor production in their flocks than their counterparts elsewhere in the UK.

Almost all confirmed reports of anthelmintic resistance in the UK have involved populations of *T. circumcincta*. However, benzimidazole resistance is also claimed to be present in *H. contortus*, *Cooperia curticei* and *Trichostrongylus* spp., and levamisole resistance in *C. curticei* and *Trichostrongylus* spp. (Abbott and others, 2004).

1.5 Current best advice aimed at reducing the development and emergence of anthelmintic resistance on UK sheep farms

1.5.1 The genetic origin of anthelmintic resistance

Alleles of genes conferring resistance to a particular anthelmintic may have pre-existed in a nematode population for a long period of time and at an extremely low frequency, even before that anthelmintic is first used (Le Jambre, 1978), but with no survival or fitness advantage compared with the vast majority of alleles conferring anthelmintic susceptibility. If this is the case, then resistance alleles should have a common origin, but depending on the age of the mutation, there could be a high level of sequence diversity of resistance alleles due to subsequent recombination. Alternatively, alleles with a selective advantage for anthelmintic resistance may arise as spontaneous mutation events during the period since the anthelmintic has been introduced. In this case, there will be less time for recombination to result in sequence diversity, and so resistance alleles will be similar. A third possibility is that resistance alleles might appear recurrently, with each event occurring in a different genetic background, leading to a variety of different alleles conferring resistance.

Once resistance alleles have emerged, then effective exposure of nematode populations within their sheep host to anthelmintic drugs kills most of the susceptible nematodes and confers a selective advantage to those nematodes having resistance alleles. Faeces produced by the sheep or goat host during the subsequent pre-patent period contain mostly eggs of the surviving resistant nematodes, which consequently contribute to a greater proportion of succeeding generations. The evolution of resistance is thus determined by the extent to which survivors of the drug treatment contribute their genes to future generations. The rate of selection for resistance is, therefore, dependent on the number of genes involved, the dominance, partial dominance or recessiveness of the alleles, and on the intensity of the selection pressure. The intensity of the selection pressure is influenced by the frequency and timing of anthelmintic treatments, and the

drug efficacy, which is in turn influenced by the dose rate, its inherent efficacy and the pharmacodynamics and pharmacokinetics within the host. The rate of selection for anthelmintic resistance is also influenced by the life expectancy and fecundity of adult nematodes, the parasite generation interval, the ability or otherwise for the parasite to self fertilise, and the proportion of the susceptible population exposed to the anthelmintic compared with that on pasture (Prichard and others, 1980).

1.5.1.1 Genetic dominance of anthelmintic resistance alleles

At low anthelmintic resistance gene frequencies, most resistance alleles are present in heterozygous nematodes, with one allele conferring resistance and one allele conferring susceptibility. If anthelmintic resistance is recessive, then the survival of alleles conferring resistance after anthelmintic treatment is poor and resistance is slow to develop. Conversely if resistance is dominant, then the survival of resistant alleles following treatment is high and the frequency in the nematode population of alleles conferring resistance increases rapidly (Leathwick and others, 2001). This explanation for the rate at which resistance develops oversimplifies the situation that occurs in the field, where several genes may be involved and dominance may be incomplete.

Benzimidazole resistance in both *H. contortus* and *T. colubriformis* appears to involve selection for two or more independent genes and is considered to be an incompletely recessive trait (Dobson and others, 1996; Roos, 1997).

Early studies indicated that levamisole resistance in *T. colubriformis* might be inherited as a sex-linked recessive trait, probably controlled by a single or cluster of tightly linked genes (Martin and others, 1988; Martin and McKenzie, 1990). However, despite being a recessive trait, levamisole resistance in *T. colubriformis* is effectively dominant in male nematodes, which only have one X chromosome. The pattern of development of levamisole resistance in a laboratory strain of *H. contortus* has been shown to be consistent with that of an autosomal recessive trait (Hoekstra and others, 1997b). This study also revealed that female fecundity and survival (fitness) was reduced in resistant

populations, suggesting that reversion to susceptibility may occur when selection ceases. This difference may partly account for the fact that resistance is relatively common in *T. colubriformis*, but rare in *H. contortus* (Sangster, 1999). This could also account for observations of a comparatively low incidence of levamisole resistance in the field and with the variable, lower level of resistance to levamisole that has been shown in UK multiple anthelmintic drug resistant field isolates of *T. circumcincta* (Bartley and others, 2004; Bartley and others, 2005; Sargison and others, 2007a).

In *H. contortus*, resistance to ivermectin appears to be inherited as a complex dominant autosomal trait, but its expression is influenced by sex in that efficacy against heterozygous (*rs*) females is lower than against heterozygous (*rs*) males (Dobson and others, 1996; Le Jambre and others, 2000). In contrast, ivermectin resistance in *T. colubriformis* appears to be inherited as a partially dominant trait under the control of multiple genes (Gill and Lacey, 1998).

1.5.1.2 Parasite reproductive biology

The effects of parasite biology such as the life expectancy and fecundity of the adult nematodes and the parasite generation interval may explain in part the propensity of *H. contortus* to develop anthelmintic resistance and why most of the early reports of anthelmintic resistance in Australia and South Africa involved *H. contortus*.

Comparison of the risk factors for selection for anthelmintic resistance in the parasitic trematode, *Fasciola hepatica* (Mitchell and others, 1998) with those for parasitic nematodes provides an example of the relevance of reproductive biology. Some populations of *F. hepatica* are triploid (Fletcher and others, 2004) and have an extra allele that confers a 50% higher rate of mutations, for example to detoxify or excrete anthelmintic drugs. Once generated, the biology of *F. hepatica* enables rapid selection for genes conferring anthelmintic resistance, both through the clonal, asexual multiplication that occurs during the sporocyst and redia developmental stages in the

intermediate host and through normal hermaphroditic self fertilisation and parthenogenetic egg production in the final host.

1.5.1.2 Gene flow

The frequency of alleles in a nematode population conferring anthelmintic resistance may also change due to gene flow, through the introduction of alleles conferring resistance or susceptibility with new sheep or goats. The impact of this would depend on the number of animals introducing resistance, the fecundity of the resistant nematodes and the subsequent management of these animals.

1.5.2 Strategies which may reduce the rate of development of anthelmintic resistance

Once resistance to an anthelmintic group has emerged within an individual sheep flock, parasitic gastroenteritis can no longer be controlled using any of the drugs belonging to that anthelmintic group. Reversion to susceptibility does not occur within flocks, even after a period of 20 years or more of not using the anthelmintic group to which resistance is present (Jackson and Coop, 2000). While lowground farmers can change from uneconomic sheep production to cereal cropping in response to unsustainable nematode control, the options for many hill and upland farmers are limited. There is therefore need for all UK sheep farmers to establish effective nematode control programmes which ensure satisfactory animal production, while preserving the efficacy of the remaining anthelmintic groups, in particular the macrocyclic lactones (Sargison, 2000b; Sargison 2002).

The probability should be considered that alleles conferring anthelmintic resistance are already present in most UK flocks, albeit at a low and clinically insignificant level. It is therefore important to ensure that these resistant alleles are not afforded any survival advantage as a result of flock nematode control practices. In practical managemental

terms, the selection pressure for anthelmintic resistance is influenced by: the frequency and timing of anthelmintic treatment; the anthelmintic dose rate; the anthelmintic drug efficacy; and the proportion of the susceptible population exposed to the anthelmintic compared with that on pasture.

1.5.2.1 Anthelmintic treatment of introduced sheep and goats

It is important to focus attention both on slowing the rate of development of anthelmintic resistance within a flock, and avoiding bringing in significant numbers of resistant nematodes through 'gene flow' with introduced sheep or goats (West and Probert, 1989; Orpin, 1991; Coles and Roush, 1992). In the absence of any sensitive, rapid and accurate diagnostic test for anthelmintic resistant nematodes in individual sheep, all introduced sheep and goats should be assumed to be sources of multiple anthelmintic resistance. All introduced sheep and goats should, therefore, be treated with an effective anthelmintic on arrival and yarded for 48 hours to ensure that any viable nematode parasite eggs have been voided before they are turned onto pastures which might be grazed by sheep within the lifespan of any hatched L_3. Ideally, introduced sheep should then be turned onto likely contaminated pasture, so that any resistant nematode parasites that survive anthelmintic treatment only make up a very small proportion of the otherwise susceptible population *in refugia*. Entry of stray sheep or goats should be prevented and basic biosecurity should be imposed to ensure that sheep or goat faeces are not brought onto the farm.

The need for quarantine anthelmintic treatment applies equally to all introduced sheep and goats, including animals returning from grazings away from home and purchased animals. Replacement rams may pose a particularly high risk because they may have been treated frequently with anthelmintics to gain a production advantage, and underestimation of their body weights is common.

The choice of anthelmintic for quarantine treatment is not straightforward. Resistance to the benzimidazole anthelmintics is already widespread, so for most flocks, the main

reason for quarantine anthelmintic treatment is to prevent the introduction of macrocyclic lactone resistant nematodes, as this group may become the only remaining useful class of anthelmintic. The current options are to use a combination of anthelmintic drugs with different mechanisms of action, or to use moxidectin.

It would be irresponsible not to promote stringent quarantine treatment of introduced sheep and goats with an effective anthelmintic or combination of anthelmintics. However, in flocks where the proportion of multiple anthelmintic resistant helminths is high, not treating introduced animals might enable introduction of useful susceptible nematode genotypes, which might dilute any resident resistant population and restore anthelmintic efficacy to a useful level (Coles, 2002). Exploitation of this strategy would depend on knowledge of anthelmintic resistance in the flocks of origin, or imposition of a quarantine period while the resistance status of the introduced animals is determined.

1.5.2.1.1 Anthelmintic drug combinations

The use as quarantine drenches of full-dose combinations of injectable macrocyclic lactones (which would also control sheep scab in the UK) and another anthelmintic with a different mechanism of action is recommended overseas (Dobson and others, 2001) and in the UK (Abbot and others, 2004). An anthelmintic drench containing a full-dose combination of albendazole, levamisole, ivermectin and praziquantel (Triton; Merial) is marketed in Australia and New Zealand, primarily for use as quarantine treatments. However, the use of proprietary anthelmintic combination drenches for nematode control is not authorised by the Veterinary Medicines Directorate (VMD) in the UK. Instead, the VMD permits sequential dosing with two different anthelmintic drugs.

Faecal egg count reduction tests that were performed in a group of Suffolk ewe lambs, one year after the confirmation of resistance to benzimidazole, imidazothiazole and macrocyclic lactone resistance in the flock, showed post treatment efficacies for ivermectin, ivermectin/benzimidazole and ivermectin/levamisole combinations against *Teladorsagia* spp. of 0, 76 and 70 percent respectively (Sargison, 2006b). These results

confirmed the presence of ivermectin resistance, and provided evidence of resistance to the two anthelmintic drug combinations. Under these situations, further selection for drug combination resistance is inevitable. Furthermore, these results indicate that full dose combinations of ivermectin and benzimidazole or levamisole anthelmintics may be ineffective as quarantine treatments in removing ivermectin resistant *T. circumcincta* from introduced sheep.

1.5.2.1.2 Moxidectin

The macrocyclic lactone group of anthelmintics includes the avermectins (ivermectin and doramectin) and milbemycin (moxidectin). These anthelmintic drugs all have structural similarities and share some mechanisms of action (Shoop and others, 1993), but differ in their potency (Pankavich and others, 1992). Moxidectin is much more liphophilic than the other macrocyclic lactone drugs, becoming concentrated in the sheep's body fat soon after administration, creating a reservoir from which they are then slowly released (McKellar and Benchaoui, 1996). The high potency of moxidectin compared with that of ivermectin, means that a much lower concentration of moxidectin is required to kill macrocyclic lactone susceptible nematode parasites. Oral formulations of moxidectin and ivermectin are both administered at a dose rate of 200 μg/kg. However, the concentration of the less potent ivermectin that is subsequently released from the sheep's fat is too low to kill nematode parasites, while that of the more potent moxidectin is sufficient, affording persistence for about 4 weeks against *T. circumcincta* and *H. contortus* (Bairden and others, 1994). This persistence is reduced in thin sheep with low body fat reserves (Lespine and others, 2004).

The presence of side resistance between the avermectins and milbemycin is illustrated by experimental efficacy studies, which have shown that 31 times the dose of moxidectin may be required to kill a population of ivermectin resistant *T. circumcincta* than to kill an ivermectin susceptible population (Shoop and others, 1993). However, the dose of moxidectin required to kill the ivermectin resistant *T. circumcincta* remained lower than the standard dose rate of 200 μg/kg, indicating that in the short term

moxidectin could be used as a quarantine drench to control ivermectin resistant nematodes (Pankavich and others, 1992). Furthermore, experimental critical efficacy studies have shown that when moxidectin is administered orally at the manufacturer's recommended dose rate of 200 μg/kg, it is more than 98 percent effective against a UK ivermectin resistant population of *T. circumcincta* (Bartley and others, 2004). Several overseas studies have shown that a dose of 200 μg /kg bodyweight of moxidectin is effective at reducing faecal worm egg counts and removing burdens of ivermectin-resistant nematodes (Kieran, 1994; Le Jambre and others, 1995; Rolfe and Fitzgibbon, 1996; Sutherland and others, 1999; Leathwick and others, 2000; Gopal and others, 2001; Vickers and others, 2001), although it has been shown that moxidectin at the manufacturer's recommended dose rate was less effective against ivermectin-resistant isolates of *Haemonchus contortus* in Australia (for example, Love and Coles, 2002; Le Jambre and others, 2005) and Brazil (Bridi and others, 1997) and against avermectin resistant isolates of *T. circumcincta* in New Zealand (for example, Hughes and others, 2004).

An attempt was made to control ivermectin resistant *T. circumcincta* in a flock of April-lambing Suffolk ewes and lambs in the south-east of Scotland (Sargison and others, 2001) using suppressive moxidectin treatments. The ewes were all orally dosed with moxidectin 10 days after turnout and again when they were removed from the pasture following weaning. All of the lambs were dosed orally with moxidectin at weaning and at 6 week intervals thereafter. The FWECs of 20 ewes, 14 days after moxidectin treatment, were all zero, while their mean FWECs 28 and 35 days after treatment were 65 epg (range 0 to 550 epg) and 230 epg (range 0 to 950 epg) respectively. All of the larvae recovered following coproculture belonged to the genus *Teladorsagia*. The FWECs of all 90 ewes about 21 days after moxidectin treatment at weaning were all zero. Oral moxidectin treatment of the lambs failed to suppress their FWECs beyond 21 days post treatment. By August, the mean FWECs of 30 lambs, 28 and 35 days after moxidectin treatment were 203 (range 0 – 2250) and 730 (range 0 – 5750) epg respectively. Coprocultures of pooled faecal material confirmed the predominance of

Teladorsagia. Scour and poor performance consistent with clinical parasitic gastroenteritis were seen in some lambs within 28 days of anthelmintic treatment. Thus, the treatment regime including oral dosing of the ewes with moxidectin to control their periparturient rise in FWECs, and suppressive oral dosing of lambs with moxidectin at 6 week intervals throughout the summer, which might have been expected to control ivermectin susceptible *T. circumcincta*, failed to prevent the establishment of significant numbers of infective larvae on the pasture and achieve the primary objective of parasitic nematode control (Sargison and others, 2005).

While oral dosing with moxidectin was apparently effective in removing adult female burdens of ivermectin resistant *T. circumcincta* from their host, positive FWECs from 21 days post treatment and the coproculture results provide evidence that the drug achieved no persistence against the UK ivermectin resistant population of *T. circumcincta* (Sargison and others, 2005).

The preservation of efficacy of moxidectin against ivermectin resistant nematode populations within the host while its persistent activity is reduced is related to the fact that the drug is effective at the manufacturer's recommended dose, but as blood concentrations fall, as occurs soon after dosing when the drug is re-distributed in the body fat reserves, they become too low to kill incoming resistant nematodes. The apparent effectiveness of oral moxidectin at a dose rate of 200 μg/kg in removing ivermectin resistant *T. circumcincta* from their host might indicate that it would be effective as a quarantine anthelmintic drench. However, the reduced persistence against *T. circumcincta* indicates the early expression of resistance (Conder and others, 1993; Ridler and others, 2002), against which background the routine use of moxidectin alone as a quarantine drench could rapidly further select for resistance through their inability to prevent incoming ivermectin-resistant larvae from establishing during the post treatment period (Sutherland and others, 1997; Le Jambre and others 1995, Sutherland and others 1999). Where the genetic inheritance of macrocyclic lactone resistance is dominant, such resistance is likely to be rapid (Le Jambre and others, 2000), so the

continued therapeutic use of moxidectin in the face of macrocyclic lactone resistance would clearly be imprudent (Rendell and others, 2006).

Therapeutic failure of oral moxidectin at a dose rate of 200 μg/kg has subsequently been reported in another Suffolk flock with a history of ivermectin resistance in the south-east of Scotland, following use of the drug for suppressive control of *T. circumcincta* (Wilson and Sargison, 2007) and in a hill Blackface flock (Sargison, *unpublished data*).

1.5.2.1.3 Current best advice for UK farmers concerning anthelmintic treatment of introduced animals

In the absence of any new anthelmintic group, the choice of anthelmintic treatment regime to reduce the risk of introduction of macrocyclic lactone resistant nematode parasites presents a dilemma. The efficacy of moxidectin as a quarantine treatment for multiple drug resistant nematodes may prove to be unsustainable, while full dose combinations of ivermectin and levamisole may be ineffective. The best current advice may be to treat introduced sheep sequentially with a full dose combination of moxidectin and levamisole, or with a combination of all three anthelmintic groups, as is routinely practised in Australia and New Zealand. Given this uncertainty surrounding quarantine anthelmintic treatments, it would be prudent to routinely monitor their efficacy (Sargison, 2006a), so as to enable the timely implementation of alternative nematode parasite control strategies, should triple resistant nematodes be introduced.

1.5.2.2 Monitoring anthelmintic efficacy

Strategies such as the use of moxidectin, sequential dosing with different classes of anthelmintics, and targeted anthelmintic treatments in response to FWECs have generally proven ineffective for the control of gastrointestinal nematode parasites in UK flocks where triple resistance was first identified as a cause of serious lamb ill thrift (Sargison and others, 2007a). The first UK flock in which triple resistance was identified (Sargison and others, 2001) has now been sold, while other affected farms have had to adapt to suboptimal productivity or are now considering fundamental,

expensive changes to their flock management, such as adoption of safe grazing, or earlier lambing and lamb creep feeding to ensure that most lambs are finished before pathogenic nematode burdens develop on pasture. Unfortunately, the sustainability of these strategies is by no means guaranteed.

In those flocks where triple anthelmintic resistance was only identified following routine monitoring, economic lamb production has mostly been sustained (Sargison, 2006a). Thus, the need to identify the anthelmintic resistance status of nematode parasites in individual sheep flocks is clear (West and Probert, 1989; McKenna, 1990).

1.5.2.3 Recommendations aimed at ensuring that nematodes are exposed to an effective anthelmintic drug concentration

Exposure of nematodes to sub-therapeutic drug concentrations increases the selection pressure for benzimidazole and imidazothiazole resistance, where the genetic basis is incompletely recessive or partially dominant. In these situations, homozygous susceptible and heterozygous partially resistant nematodes are killed at the full therapeutic dose, while homozygous resistant nematodes survive (Martin, 1989). Sub-therapeutic drug concentrations enable heterozygous partially resistant nematodes, which are initially more common than homozygous resistant nematodes to survive (Silvestre and others, 2001), thus increasing the frequency of resistant alleles in subsequent populations.

1.5.2.3.1 Under dosing

Under dosing due to inaccurate judgment of sheep bodyweights and faulty dosing guns is commonplace. While this may have little immediate economic effect on animal production, it inevitably selects for anthelmintic resistance (Waller, 1986; Martin, 1989). Poor drenching technique, miscalculation of the correct dose volume and use of inaccurate weigh scales compounds the problem (Besier and Hopkins, 1988). Most benzimidazole and macrocyclic lactone drenches have a wide safety margin so if necessary, it is preferable to overestimate the required dose volume.

Under dosing may also arise following incorrect storage of anthelmintic drugs, use of expired product, mixing incompatible drugs or chemicals before dosing, or use of products of dubious origin (Waller and others, 1996; Čerňanská and others, 2006).

1.5.2.3.2 Drug bioavailability

The efficacy of drugs against nematode parasites can be altered by the effects of disease or management on the physiology of their host. Anthelmintic bioavailability may be reduced due to rapid flow of digesta through the intestines of scouring lambs (Hennessy, 1994), for example the efficacy of the relatively insoluble benzimidazole anthelmintic, mebendazole, is reduced in lambs with nematodirosis (Sargison, 2006c). The bioavailability of drugs such as the fasciolicidal benzimidazole, triclabendazole, that are altered to active sulfoxide metabolites in the liver, is reduced in animals with severe liver disease, for example associated with subacute fascioliasis (Robinson and others, 2004).

The efficacy of benzimidazole and oral macrocyclic lactone anthelmintics is dependent on the duration of nematode exposure to a therapeutic drug concentration (the efficacy of levamisole is determined by the initial concentrations achieved (Coles and others, 1974)), and can be enhanced by prolongation of the drug's plasma concentration profile. Within two hours of oral administration, benzimidazole and macrocyclic lactone anthelmintics become largely associated with particulate digesta in the ruminoreticulum (Hennessy and others, 1994). Mixing of drug-associated digesta fluid and particulate matter in the rumen and outflow through the reticulo-omasal orifice to the abomasum results in the average half life of digesta fluid and particulate material being 12 hours and 18 hours respectively (Hennessy, 1993a), although this is influenced by the animal's diet and feeding management (Taylor and others, 1992). The residence time of the drug-digesta complex in the ruminoreticulum and the subsequent flow rate of the digesta through the gastrointestinal tract contribute significantly to the rate of drug absorption and recycling (Ali and Hennessy, 1995a) and to the duration of anthelmintic availability

(Ali and Chick, 1992). The duration of the particulate digesta associated anthelmintic reservoir is determined by the rate of flow of digesta from the ruminoreticulum, which is influenced by feed type, being shorter for fresh green feed compared with conserved rations (Ali and Chick, 1992), but also responds quickly to a reduction in feed intake (Taylor and others, 1992), and is about halved within 24 hours of halving the feed intake (Ali and Hennessy, 1995b). Thus a significant interaction exists between feed intake and anthelmintic efficacy. In some circumstances, reducing feed intake by yarding for 24 hours before and 8 hours after drenching extends the plasma profile of benzimidazole (Ali and Hennessy, 1995a; Ali and Hennessy, 1995b; Ali and others, 1995; Hennessy and others, 1995) and macrocyclic lactone drenches (Ali and Hennessy, 1996). In the presence of resistant nematode parasites, this may reduce selection for anthelmintic resistance (Hennessy, 1993a).

Oxfendazole has greater solubility in abomasal fluid than in ruminal fluid, due to the lower pH in the abomasum (Marriner and Bogan, 1981; Marriner and others, 1985). Oxfendazole is, therefore, rapidly absorbed if it bypasses the ruminoreticulum due to closure of the reticular groove, resulting in slightly higher initial blood concentrations. However, the absence of a rumen reservoir may result in significantly reduced plasma concentrations with time, despite the initially higher circulatory drug concentrations (Prichard and Hennessy, 1981). Orally administered benzimidazoles provide some direct toxic effect to parasites in the gut lumen, however significant parasite-drug contact comes from exchange of absorbed metabolites across the gastrointestinal tract wall (Hennessy, 1993b). Soluble metabolites are absorbed from the small intestine to the systemic circulation. In the liver, benzimidazole metabolites may be conjugated or remain unconjugated, and a significant portion of each form is secreted in the bile (Hennessy and others, 1992). Unconjugated metabolites are reabsorbed from the small intestine, while the conjugated benzimidazole metabolites undergo bacterial hydrolysis in the large intestine, promoting further absorption. Benzimidazole metabolites secreted in bile are thus enterohepatically recycled and delivered to many varied sites.

Reticular groove closure is hypothesised as a cause of erratic behaviour of some benzimidazole drenches (Hogarth-Scott and others, 1976). This hypothesis is supported by the demonstration of reduced bioavailability and efficacy of the less soluble benzimidazole drugs (fenbendazole and parbendazole) against resistant nematodes following direct administration to the abomasum (Kelly and others, 1977; Prichard and others, 1978). Furthermore, the pharmacokinetic efficacy of oxfendazole is enhanced when the drug is administered directly into the rumen, rather than into the abomasum (Prichard and Hennessy, 1981). Ruminoreticulum bypass is, therefore important as a factor influencing the rate of development of resistance (Prichard, 1985; Taylor and others, 1992).

Under certain grazing conditions, for example where sheep are grazed on lush green pasture, their ruminoreticulum contents become fluid after yarding for a period of 24 hours (Clunies Ross, 1934). This has been shown to result in closure of the reticular groove following oral dosing in about 35 percent of weaned lambs, diverting the dose into the abomasum rather than the ruminoreticulum and potentially reducing the efficacy of benzimidazole or macrocyclic lactone anthelmintics (Sargison, 1997; Sargison and others, 1998). Thus the relative effects of yarding before anthelmintic dosing on the rate of passage of ruminoreticulum contents and of reticular groove closure with regards to anthelmintic efficacy is uncertain.

The incidence of ruminoreticulum bypass is reduced when the lambs, which had been yarded for 24 hours, are dosed with a low volume drench (Sargison and others, 1998), supporting the use of low volume drench formulations as a management tool to mitigate against ruminoreticulum bypass of benzimidazole or macrocyclic lactone anthelmintics (Benchaoui and others, 1995; Hennessy and others, 1997).

1.5.2.3.3 Avoid keeping sheep and goats on the same farm

Sheep and goats both serve as natural hosts for many of the same pathogenic nematode parasites, in particular *H. contortus*, *T. circumcincta*, *T. colubriformis* and *T. vitrinus*. Cross infection of multiple drug resistant strains from goats to sheep has long been

considered a significant risk to sheep (Watson, 1994). Field strains of macrocyclic lactone resistant *T. circumcincta* (Watson and others, 1996) and *T. colubriformis* (Gopal and others, 1999) isolated from goats have been found to be equally infective for sheep, and ivermectin resistance has been confirmed in sheep following experimental passage from goats (Gopal and others, 1999).

Intrinsic differences exist between the pharmacokinetics of anthelmintic drug absorption and elimination in goats and sheep. Goats metabolise anthelmintic drugs more rapidly than sheep (Chartier and others, 1998) and may also show a higher incidence of ruminoreticulum bypass, reducing the effective drug concentration (Waller and others, 1986). Furthermore, adult goats do not become fully immune to nematodes, perhaps associated with nematode evolution in response low levels of parasite challenge when goats are kept in their natural environment (Jackson and others, 1991). As a result, when goats are farmed and forced to graze, rather than naturally browse, they generally develop and sustain much higher nematode parasite burdens than sheep that are exposed to the same level of challenge. Unlike the situation in adult female sheep, which only transiently harbour significant nematode parasite burdens during their periparturient period of lowered immunity, adult goats can harbour high nematode parasite burdens throughout their production cycle. These high nematode parasite burdens seldom result in such severe disease as equivalent burdens in sheep, because the severity of clinical disease is related to the host's immune response. However, the combined effects of exposure of a larger proportion of the nematode parasite population to anthelmintic at the time of treatment, inherently lower anthelmintic drug efficacy, and a lower contribution of host immunity to re-enforce anthelmintic efficacy, impose a significantly higher selection pressure for anthelmintic resistance in goats than in sheep (Hennessy, 1994).

Resistance in individual parasitic nematode species to each class of anthelmintic drug has been reported globally in goats, several years before the first field report in sheep. For example in New Zealand, ivermectin resistance in *T. circumcincta* was first reported

in goats in 1988 (Watson and Hosking, 1990), 11 years before the first field report in sheep. The problem in goats in New Zealand is now widespread, predominantly involving *T. circumcincta* (Badger and McKenna, 1990; Leathwick, 1995; Watson and others, 1996). Many reports have involved co-resistance to all three broad spectrum anthelmintic classes (McKenna and others, 1990; Pomroy and others, 1992). Therapeutic failure of moxidectin against *T. circumcincta* in goats is now commonplace (Pomroy and Whelan, 1993; Leathwick, 1995; Watson and others, 1996). The prevalence and degree of multiple resistance to benzimidazole, imidazothiazole and macrocyclic lactone anthelmintics in *H. contortus* is reported to have reached crisis point in South African goat herds and is now emerging as a serious problem in sheep (Van Wyk and others, 1999).

T. circumcincta resistance to ivermectin was first reported in goats in Scotland in 1992 (Jackson and others, 1992b), 9 years before the first report in sheep (Sargison and others, 2001). Ivermectin resistant *T. circumcincta* are now widespread in goats throughout Europe, having been reported in France (Chartier and others, 1998), Spain (Álvarez-Sánchez and others, 2001), Switzerland (Schnyder and others, 2005) and Denmark (Maingi and others, 1997).

1.5.2.4 Recommendations aimed at the timing and frequency of anthelmintic treatment and ensuring that only a small proportion of the nematode population is exposed to the anthelmintic

The rate of selection for anthelmintic resistance by a nematode parasite species is influenced by the proportion of its total population which is exposed to the drug (Prichard and others, 1980). The greater the proportion of the nematode population exposed to the drug in its sheep host compared to that on pasture at the time of anthelmintic treatment, the faster the selection for resistance. Thus, the rate of selection for anthelmintic resistance is inversely proportional to the percentage of the total parasite population that is on pasture as eggs and developing larvae (referred to as *in refugia*) at the time of treatment (Van Wyk, 2001; Coles, 2002). If the proportion of free

living nematode parasites at the time of anthelmintic treatment is large, then the offspring of resistant nematodes are diluted, but if the nematode population *in refugia* is small, then the offspring of resistant nematodes will constitute a larger proportion of the next generation (Taylor and Hunt, 1988). Similar circumstances occur naturally, for example in winter rainfall areas of Australia, where during the hot, dry summer, few nematode eggs or larvae survive on pasture and the practice of anthelmintic drenching during the summer (Anderson, 1972) leads to the rapid appearance of anthelmintic resistance (Prichard and others, 1980). The relationship between the size of the nematode population *in refugia* and that in its host is further influenced by factors which affect the survival and rate of development of the free living nematode stages. The objective of nematode control is to limit exposure of susceptible sheep to infective larvae on pasture. Thus, the rate of selection for anthelmintic resistance is highest on those farms which achieve the most effective nematode control. Some guidelines aimed at slowing the emergence of anthelmintic resistance, therefore, inevitably involve a compromise between achieving adequate nematode parasite control and reducing the rate of selection for anthelmintic resistance.

The low prevalence of benzimidazole resistance in sheep nematode parasites in some southern European countries, despite long-term reliance on the drug is probably related to the ineffective *ad hoc* use of anthelmintics and failure to achieve adequate nematode parasite control (Papadopoulos and others, 2001; Álvarez-Sánchez and others, 2001). Occasional curative treatments given only when clinical disease is suspected probably select least for resistance, because only a very small proportion of the parasite population would be exposed to the drug (Le Jambre, 1978). By contrast, in southern Latin America, where anthelmintic resistance is widespread, warm and humid conditions favour parasite development and transmission throughout the year. Consequently, anthelmintic treatments are required every three weeks throughout the year in some regions, to avoid sheep deaths due to haemonchosis (Waller and others, 1996). Under these situations, the balance between achieving adequate nematode parasite control and avoiding selection for anthelmintic resistance may be impossible to achieve.

1.5.2.4.1 Extend the interval between anthelmintic treatments

Suppressive control of nematode parasites aimed at preventing pasture contamination with eggs and developing larvae, frequently involves conventional anthelmintic treatment of susceptible sheep at intervals close to the parasite's pre-patent period, which had initially afforded good nematode control (Barton, 1983). The costs of anthelmintics are low (especially in Australia and New Zealand) when compared with the potential return from their use and, within limits, production per sheep increases with increased frequency of drenching (Johnstone and others, 1979). It is therefore not surprising that traditionally farmers have adopted high frequency drenching programmes with treatment intervals close to the prepatent period of nematode parasites. This high frequency treatment strategy has been an important factor in the emergence of anthelmintic resistance worldwide (Donald and others, 1980; Martin and others, 1982; Barton, 1980; Barton, 1983, Sargison and others, 2007a). Australian modeling studies have suggested that high anthelmintic treatment frequencies may be the principal source of selection for resistance (Barnes and others, 1995; Dobson and others, 1996). Anthelmintic usage and selection for resistance could be reduced by extending the suppressive conventional anthelmintic treatment interval, although this strategy may not prevent production losses due to parasitic gastroenteritis. This dilemma might be addressed by basing the timing of anthelmintic treatments on monitoring of FWEC data. However the success of this strategy depends on the level of parasite challenge, the stocking density, geography and weather conditions on individual farms. During spring and early summer on heavily stocked farms, lamb FWECs can increase rapidly, so substantial pasture contamination may already have occurred by that time that intervention targets are identified. FWEC monitoring can be useful to determine the optimal timing of suppressive anthelmintic treatments on some farms where the level of pasture larval challenge is likely to be low, as a result of summer drought conditions or low stocking densities, but intervention targets must be based on previous knowledge of the epidemiology of parasitic gastroenteritis in the individual flock. Thus, the principal

reason for monitoring FWECs is to establish the pattern of pasture larval contamination on individual farms, providing a basis for future nematode control strategies.

1.5.2.4.2 Avoid unnecessary anthelmintic treatments

Reducing the reliance on anthelmintics by removing unnecessary treatments, while maintaining good control of parasitic gastroenteritis, is intuitive. In many flocks, ewes are treated with an anthelmintic before mating in the belief that the practice might improve lambing percentages (Sargison and Scott, 2003). The benefits of this strategy are that if the ewes are in poor body condition, anthelmintic treatment before mating can lead to improved lambing percentages, provided that they are subsequently well fed. Anthelmintic treatment before mating can also provide some insurance against the effects of the possible introduction of new nematode parasite species. However, the disadvantages associated with anthelmintic treatment of ewes before mating are that it does not lead to improved lambing percentages where ewes are already in good body condition, incurring unnecessary expense and effort (Houdijk and others, 2001). Furthermore the practice possibly selects for anthelmintic resistance by exposing parasites to anthelmintics at a time when a significant proportion are in a hypobiotic, hard-to-kill state (Michel, 1967; McKenna, 1974; Reid and Armour, 1975b; Prichard, 1988), and by affording surviving resistant nematodes with a prolonged period during which they dominate egg production in immune sheep. It is therefore impossible to make general recommendations about anthelmintic treatment of ewes. Risks must be assessed for individual flocks. The decision can be aided by monitoring of ewe FWECs, although these are difficult to interpret without previous knowledge of the flock. For example, in some cases low counts can become significant when the total daily faecal output of ewes is taken into account. In other cases, high counts may be insignificant due to the predominance of less pathogenic large intestinal nematodes, or the fact that larvae hatched from eggs shed by immune ewes may be less fit, and therefore less likely to reach the L_3 stage, than those shed by naive sheep (Jørgensen and others, 1998). In some cases, the best strategy may be only to treat lean ewes before mating. This

strategy avoids unnecessary anthelmintic usage and permits limited pasture contamination by eggs of susceptible nematodes shed by the untreated ewes.

Treatment of lambs may be unnecessary when they are grazed on safe pasture, or later in the year when they have acquired some immunity. In these situations, the decision to treat lambs or not, can be supported by monitoring of FWECs.

1.5.2.4.3 Integrated nematode parasite control

Reliance on anthelmintics may be reduced by the use of safe grazing. On many farms, the opportunity exists to introduce safe grazing for a proportion of the sheep flock by alternating annually between arable cropping or cattle grazing and sheep grazing. However, while the use of safe grazing can provide excellent control of parasitic gastroenteritis in the short term, the practice selects for anthelmintic resistance when it is integrated with anthelmintic treatments, such as in a traditional dose-and-move system (Taylor and Hunt, 1988). This system results in a situation whereby the new pasture is contaminated only with nematodes which survive anthelmintic treatment and are then afforded a prolonged period of reproductive advantage over susceptible nematodes (Martin and others, 1985). While the subsequent level of pasture larval contamination remains low, providing excellent short-term nematode control, the nematode population *in refugia* becomes predominantly anthelmintic resistant. Over a period of time, these anthelmintic resistant nematodes are multiplied in susceptible sheep that graze on the pasture, resulting in potentially hard to manage parasitic gastroenteritis.

All of the recommendations concerned with anthelmintic treatments before a move onto safe pasture involve a compromise between long-term nematode control and selection for anthelmintic resistance. This risk of selection for anthelmintic resistance may be reduced by leaving a proportion of the flock untreated. It may be possible to target anthelmintic treatments to scouring or lighter weight lambs, leaving faster growing animals untreated. Alternatively, the move onto safe grazing can be delayed for about 5 days after conventional anthelmintic treatment, so that both resistant nematodes, and

susceptible nematodes acquired during this period are carried onto the safe grazing. When anthelmintic treatment is considered essential before a move to safe grazing, sheep grazing of the pasture should be avoided during the following season.

1.5.2.4.4 Targeted selective treatments

Individual sheep differ in their tolerance to the same level of infective larval challenge, with some animals suffering reduced production and developing high FWECs, while others develop low FWECs (for example, Greer, 2008). In South Africa, where natural selection for tolerance to haemonchosis has occurred over a period of several decades, less than 30 percent of the animals in some flocks may develop clinical signs of disease and contribute significantly to pasture contamination. An effective system referred to as FAMACHA©, has been tested in South Africa whereby anthelmintic treatments are targeted to members of the flock that are least tolerant to haemonchosis, based on the monitoring of conjunctival colour for signs of anaemia (Van Wyk, 2008). Using this system the number of anthelmintic treatments can be substantially reduced, while maintaining a reasonable level of flock productivity. Studies in France have shown that the highest milk producing goats have highest FWECs and that milk production can be used as an indicator for targeted selective anthelmintic treatment (Hoste and others, 2002). When applied to flocks or herds with a high proportion of nematode tolerant animals, these approaches ensure a reasonable level of parasite control, substantially reduce anthelmintic treatment costs, and importantly ensure that a population of susceptible nematodes is maintained *in refugia*.

When UK sheep are exposed to *T. circumcincta* infective larvae, individual animals show different liveweight gain responses, with the least tolerant subsequently developing the highest FWECs (Frank Jackson, *personal communication*). However, changes in liveweight gain can be detected before FWECs increase, providing a potential indicator for targeted selective treatments (Sykes and Coop, 1976; Coop and others, 1982). Field studies have been performed comparing targeted selective treatments based on fortnightly weighing to determine changes in liveweight gain with a

four-weekly neo-suppressive anthelmintic treatment regime (Jackson and others, 2007). Preliminary results suggest that while targeted selective treatments may result in slightly poorer short term productivity than neo-suppressive treatments, they select more slowly for anthelmintic resistance (Jackson and others, 2007).

The rate of development of anthelmintic resistance varies inversely with the proportion of the nematode population *in refugia* at the time of treatment (Van Wyk, 2001). Targeted anthelmintic treatment regimes may, therefore prove to be essential in order to preserve anthelmintic efficacy in UK sheep flocks. However such approaches based on maintaining a susceptible nematode population *in refugia* are more complex than conventional whole flock treatment regimes, and are only likely to be adopted if their theoretical basis is understood, or they are shown to be valid.

1.5.2.4.5 Anthelmintic treatment of periparturient ewes

Successful control of gastrointestinal nematode parasitism in lambs, generally starts with anthelmintic treatment of ewes at lambing to limit the periparturient rise in FWECs (Salisbury and Arundel, 1970; Gibson, 1973; Herd and others, 1983). A single oral treatment at lambing with moxidectin, removes nematode parasites from the ewes and enables them to remove over-wintered infective larvae from the pasture without themselves further contributing to subsequent pasture *T. circumcincta* contamination for a period of about 6 weeks (Taylor and others, 1993). Under certain circumstances, this practice may select strongly for macrocyclic lactone resistance. UK pasture burdens of over wintered infective larvae vary from year to year, associated with environmental temperature and humidity, the nematode burden accumulated over the previous grazing season, and the period for which the pasture was rested from *T. circumcincta* parasitised sheep (Sargison and others, 2002). While the population of over wintered infective larvae *in refugia* can be very high, it may also sometimes be extremely low, incurring a high selection pressure for resistance when periparturient ewes are unnecessarily dosed with moxidectin before moving onto pasture. Selection for resistance by moxidectin at the time of dosing would have been unlikely, due to its high efficacy against resident

adult nematodes, but its poorer efficacy against fourth stage larvae with ivermectin resistant genotypes (Bartley and others, 2004) may enable selection for resistance during the protection period after drug administration (Pomroy, 1998; Le Jambre and others, 1999a; Barnes and others, 2001).

1.5.2.4.6 Annual drench rotation

The use of a different broad spectrum anthelmintic group each year has been widely promoted (Prichard and others, 1980; Taylor and Hunt, 1989; Coles and Roush 1992). However, epidemiological evidence and field data to support positively this strategy are not currently apparent. Mathematical modeling simulating control of *T. colubriformis* under Australian conditions by two anthelmintic drug groups with independent resistance genes, indicated that in the long term, there was no difference in the rate of evolution of resistance between strategies involving rotation at each drench, annual drench rotation, five-, or ten-year rotation (Barnes and others, 1995). In the absence of reversion to anthelmintic susceptibility, the presence of resistance was merely hidden when the alternative anthelmintic was being used.

While annual drench rotation is not contraindicated and may indeed be shown to be beneficial under UK conditions, the perceived need to adhere to an annual anthelmintic group rotation may interfere with good nematode parasite control practice, for example involving the choice of anthelmintic treatment for quarantine treatment of introduced sheep or periparturient ewes. Rather than adhering strictly to the annual rotation of anthelmintic groups, consideration should be given to ensuring that the most appropriate drug is used for each anthelmintic treatment.

Periodic use of narrow spectrum anthelmintics has been recommended to slow the evolution of anthelmintic resistance. The "Wormkill" programme for the control of *H. contortus, Trichostrongylus* spp. and *T. circumcincta* in summer rainfall areas of coastal Australia, includes control of *H. contortus* by 1 to 3 spring and summer treatments with the narrow spectrum salicylanalide derivative drug, closantel (Dash 1986).

Trichostrongylus spp. and *T. circumcincta* are controlled by strategic summer-autumn treatment with broad spectrum anthelmintics (Barger and others, 1991). Unfortunately, control regimens incorporating salicylanilides such as closantel, are only effective against haematophagous parasites, and are of limited relevance in the UK.

1.5.2.5 Selection for anthelmintic resistance by the use of injectable macrocyclic lactones as systemic endectocides

The risks of emergence of anthelmintic resistance in the UK may be compounded by the high prevalence of psoroptic mange in sheep (sheep scab), that necessitates the frequent, routine use on many farms of injectable macrocyclic lactones as systemic endectocides (Sargison and others, 1995b; Sargison and others 2006b). Increasing numbers of UK sheep farmers now rely solely on the use of macrocyclic lactone injections for the control of sheep scab (Sargison and others, 1995a), due to the high prevalence of the disease and burgeoning environmental concerns, regulatory expense and awareness of human health problems associated with plunge dipping. It has become commonplace for some farmers to treat their sheep flocks with a macrocyclic lactone injection up to four times per year, between September and May, in often unsuccessful attempts to control sheep scab (Sargison and others, 2007c). There is now concern that this level of macrocyclic lactone systemic endectocide increases the selection pressure on anthelmintic resistant parasitic nematodes.

1.5.2.5.1 Selection for resistance by injectable formulations of macrocyclic lactone systemic endectocides

The macrocyclic lactone anthelmintics can be administered to sheep both orally and systemically, but are only fully effective against *Psoroptes ovis* mites when administered by subcutaneous (ivermectin and moxidectin) or intramuscular (doramectin) injection. The pharmacokinetics of oral and injectable macrocyclic lactone drugs differ. The most efficient method of administration in terms of drug bioavailability is by injection (Alvinerie, 1997), leading to higher systemic drug concentrations, which closely parallel concentrations at target tissues (Marriner and others, 1987; Alvinerie and others, 1998),

and a longer period of activity against re-infection (Borgsteede, 1993). However, macrocyclic lactones appear to be more effective against both susceptible and resistant *T. vitrinus* (Borgsteede, 1993), *T. circumcincta* and *T. colubriformis* (Gopal and others, 2001; Alka and others, 2004) when given orally compared to the subcutaneous route. This apparent conflict between the pharmacological observations of higher tissue concentrations and field observations of lower efficacy of injectable macrocyclic lactones is probably related to the importance of initial exposure of nematodes in the abomasum and proximal small intestine within a few hours of administration to the orally administered drug (Lespine and others, 2005). Conversely, the treatment of animals harbouring macrocyclic lactone resistant nematodes with an injectable macrocyclic lactone formulation will allow resistant nematodes to survive and could promote the development of resistance. Therefore, while injectable macrocyclic lactone formulations are often used as a combined quarantine treatment for sheep scab and nematodes, oral formulations may be more appropriate to avoid the introduction of anthelmintic resistant nematodes (Gopal and others, 2001).

1.5.2.5.2 Selection for anthelmintic resistance by the persistent anthelmintic effects of systemic endectocides

The persistence of injectable formulations of avermectin drugs that are used as systemic endectocides is longer than that of the oral formulations (Borgsteede, 1993). There is evidence that persistent anthelmintics, can select for resistance through their inability to prevent incoming ivermectin-resistant larvae from establishing during the post treatment period (Le Jambre and others, 1995; Sutherland and others, 1999). The longer this period, the greater the reproductive advantage and contribution of resistant genotypes to the pool of predominantly susceptible genotypes on pasture (Dobson and others, 1996). Furthermore, during the period of persistent activity, incoming larvae with susceptible genotypes cannot establish in the host, while some those with resistant genotypes can (Sargison and others, 2005). Thus the use of persistent systemic endectocides for the control of sheep scab enables both 'head' selection, acting on the nematode parasites present in the sheep at the time of treatment (which occurs with all anthelmintic drugs)

and ‘tail’ selection for resistance, and on incoming larvae during the period of persistent activity (Dobson and others, 1996). However, the relative importance of ‘head’ and ‘tail’ selection depends on the epidemiology of the parasite in the specific production system, the frequency of persistent anthelmintic treatment, and timing of treatment. For example, ‘tail’ selection only occurs in resistant incoming larvae, so is of little significance in situations where animals are moved to safe pasture after treatment (Dobson and others 1996), but potentially more important when animals are returned to contaminated pasture after treatment (Leathwick and others, 2001).

1.5.2.5.3 Selection for anthelmintic resistance due to the timing of systemic endectocide treatments

Systemic endectocides are routinely administered to adult sheep that have acquired immunity to nematode parasites. Treating immune adult sheep can result in greater selection pressure for resistance than treating lambs in which immunity is not yet fully developed, because an immune host is able to prevent all but a few susceptible larvae ingested after treatment from establishing and diluting surviving resistant nematodes (Le Jambre and others, 1999a; Smith and others, 1999). Furthermore, a significant proportion of the nematodes present in adult sheep hosts during the autumn and winter when systemic endectocide treatments for sheep scab control are regularly administered may be hypobiotic, hard-to-kill EL_4, increasing the risk of ‘head’ selection for anthelmintic resistance. In some circumstances, the combined effects of anthelmintic persistence, host immunity and the parasite stages present at the time of treatment could, therefore, combine to result in high levels of both ‘head’ and ‘tail’ selection for anthelmintic resistance by the use of systemic endectocides for the control of sheep scab.

1.5.3 Management of parasitic gastroenteritis in the presence of anthelmintic resistance

There are no straightforward solutions for the management of parasitic gastroenteritis in the face of high levels of multiple anthelmintic drug resistance. There are differences between farms in terms of environment, nutrition, management and nematode population size, composition and resistance. Nematode parasite control programmes therefore need to be developed for individual flocks, based on an understanding of their epidemiology, control methods and of the principles involved with anthelmintic resistance.

In most flocks, production losses to parasitic gastroenteritis cannot be prevented without some reliance on anthelmintic drenches, although sustainable control of nematode parasites depends on the development of methods which do not rely totally on anthelmintics. Practical solutions for UK flocks might include: creep feeding or provision of alternative forage crops to finish lambs early, before pasture infective larval burdens become production limiting; development of strategies involving preparation of pastures for susceptible sheep using cereal crops or prior grazing with cattle or older ewes; manipulation of strategies to increase the proportion of anthelmintic susceptible nematodes *in refugia*; development of molecular or serological tests which might help to select for host resistance or resilience to nematode parasites; or manipulation of nutrition and body condition scores to enhance natural immunity.

Full dose anthelmintic combination drenches can extend the application of the drugs involved when compared to their use independently and have proven useful overseas for the management of parasitic gastroenteritis on farms with benzimidazole resistance (Anderson and others, 1988).

In the short term, benzimidazole anthelmintics may become more effective against resistant nematodes when the parasite is exposed to an effective concentration of the drug for a

longer period. Similar principles apply to macrocyclic lactone anthelmintics, but not levamisole or morantel. Two to three full anthelmintic doses, repeated 12 hours apart for benzimidazoles, or 36 to 48 hours apart for ivermectin (Hennessy 1994), have substantially better efficacy against resistant nematodes than a single treatment at double or treble the recommended dose rate (Barger, 1995). However, this practice is unlikely to prove to be practical or sustainable.

Controlled release albendazole (Anderson and others, 1980) and ivermectin (Gogolewski and others, 1997) capsules are not available for use in sheep in the UK, but have been widely used overseas. The controlled release capsules prolong the equilibrium between anthelmintic and tubulin. The anthelmintic efficacy against adult resistant nematodes is slightly improved, but the main advantage of the controlled release capsules is against more sensitive incoming larvae. When benzimidazole resistance is present, parasitic gastroenteritis can be controlled in lambs by treatment with an alternative effective anthelmintic at the same time as bolus administration. The effective anthelmintic removes the adult benzimidazole resistant parasite population, while the controlled release albendazole is effective against subsequent pasture acquired larvae (Barger, 1993). In ewes, controlled release capsules are most effective if given pre-lambing when the parasite burdens are low. However, a number of studies have shown that controlled release ivermectin capsules potentially select rapidly for macrocyclic lactone resistance due to the ability of resistant adults to survive treatment and of resistant larvae to establish during the entire period of persistency, while susceptible larvae are excluded (Dobson and others 1996; Le Jambre and others, 1999a; Sutherland and others, 2000; Barnes and others, 2001).

1.6 Diagnostic markers for anthelmintic resistance

1.6.1 Current tests for anthelmintic resistance

Most recommended management practices aimed at delaying the onset of anthelmintic resistance are based on theoretical principles, or on practical experience of anthelmintic resistance in Australia, New Zealand and South Africa, where sheep management, and the epidemiology of parasitic gastroenteritis, may not reflect the situation in individual flocks in other countries such as the UK. There is therefore a need for sensitive diagnostic tests that can be applied to monitor and develop control strategies aimed at maintaining the useful life of the currently available drugs, in particular the macrocyclic lactones. For example, if it becomes apparent that anthelmintic resistance in UK sheep flocks is primarily caused by gene flow, then the most important advice will be to ensure effective quarantine anthelmintic treatment of introduced animals. Alternatively, if anthelmintic resistance is shown to be due to pre-existing mutations or a high rate of independent spontaneous mutations, then management strategies aimed at maintaining susceptible nematode populations *in refugia* will be paramount.

1.6.1.1 Drench check

Monitoring the FWECs of about 10 sheep, within the prepatent period following anthelmintic treatment is widely used to provide a practical indication of treatment efficacy. However, the test cannot differentiate between inefficacy due to poor drenching practice and anthelmintic resistance.

1.6.1.2 Faecal egg count reduction test

The faecal egg count reduction test (FECRT) provides a practical, reliable, on-farm diagnostic test for resistance to each of the anthelmintic groups, while compensating for other causes of poor anthelmintic efficacy. Post treatment efficacies are calculated from the arithmetic mean pre- and post-treatment FWECs of about 10 sheep (Coles and others, 1992; Coles and others, 2006). The sensitivity of the test is improved when the

pre-treatment FWECs are high, or by using pre- and post-treatment coprocultures and larval differentiation to ascribe resistance to trichostrongyle genera and define resistant populations more accurately (McKenna, 1996). Inaccuracies need to be considered, associated with differential hatching of different genera and attributing percentage generic compositions to post treatment samples where the faecal trichostrongyle egg count is low (McKenna, 1997). Use of a discriminating half dose of ivermectin has been proposed as another method to improve the sensitivity of the undifferentiated FECRT (Palmer and others, 2001), although underdosing lowers the test's specificity (Van Wyk and others, 1997b). However, even these modified FECRTs are inadequate for the identification of the presence of a low frequency of resistant nematodes for the identification of emerging resistance, or to monitor subtle changes in the level of anthelmintic resistance (Martin and others, 1989). For example, in Australia, experimental studies showed that benzimidazole resistance in both *T. colubriformis* and *T. circumcincta* could not be unequivocally detected using a FECRT until the frequency of resistant genotypes in the population approached 50 percent (Martin and others, 1989), a level at which continued rapid emergence of resistance is inevitable following further use of that anthelmintic.

1.6.1.3 Critical efficacy test

The 'gold standard' confirmation of anthelmintic resistance depends on artificial infection of nematode-free sheep, anthelmintic treatment, humane slaughter and performance of postmortem total nematode counts. This is important for the characterisation of nematode strains, but the test is impractical as a method to monitor changes in the level of anthelmintic resistance in the field.

1.6.1.4 In vitro *bioassays*

Various bioassays have been developed for the diagnosis of anthelmintic resistance. The egg hatch assay (EHA) which monitors the effect of *in vitro* benzimidazole exposure on the development of nematode eggs (Le Jambre, 1976) is relatively straightforward to perform and reasonably consistently accurate for the diagnosis of benzimidazole

resistance. Adaptations of this assay have less reliably been used for the assessment of levamisole resistance (Dobson and others, 1986). The larval development test (LDT) monitors the effects of benzimidazole and levamisole on the development of eggs to L_3, and is used for the diagnosis of benzimidazole and levamisole resistance (Coles and others, 1988; Lacey and others, 1990). However, the test is cumbersome and results are sometimes inconsistent.

The LDT can be used to provide an indication of the presence of ivermectin resistance in some, but not all field strains *H. contortus*, where a high level of anthelmintic resistance has been demonstrated *in vivo* (Gill and others, 1995; Kotze and others, 2002). However, the sensitivity of the LDT has proved to be insufficient for the identification of resistance in larval stages of laboratory selected resistant strains of *H. contortus* (Gill and others, 1998), raising questions about potential involvement of different resistance mechanisms between field and laboratory selected strains.

Assays such as the larval feeding inhibition assay (LFIA), larval migration inhibition assay (LMIA) (Kotze and others, 2006a) and adult motility assay (AMA) have been developed to monitor the *in vitro* response of nematodes to anthelmintics that induce paralysis (Gill and others, 1991; Wagland and others, 1992) and are useful as research tools for the study of macrocyclic lactone resistance. These assays are highly sensitive to complex, unresolved extrinsic factors and the difference in discriminatory doses between anthelmintic sensitive and resistant nematode populations is small, potentially resulting in inaccuracy.

The value of *in vitro* bioassays for the study of macrocyclic lactone resistance in the field is further complicated by the fact that their target mechanisms of anthelmintic resistance may differ from those involved within the sheep host. For example, the LFIA measures the effects of ivermectin on the pharyngeal pumping mechanism of L_1, a stage that is not intentionally exposed to anthelmintic drugs in the field. The LMIA and AMA involve *in vitro* exposure to concentrations of ivermectin that greatly exceed those that

occur *in vivo* within the nematode parasites' host (*personal observation*). Thus, the results may not represent the true anthelmintic resistance status of the field nematode population. Nevertheless, *in vitro* assays are useful for the comparison of resistance phenotypes between monospecific isolates when they are conducted at similar times and when nematodes are collected from similarly managed donor animals.

1.6.2 The need for molecular markers for anthelmintic resistance

Unfortunately, the sensitivity or practicality of current non-genetic tests for anthelmintic resistance (Martin and others, 1989) is inadequate for the detection of subtle changes in the frequency of resistant genotypes associated with different management practices. Sensitive, molecular tests are therefore required for the accurate and empirical assessment of management practices that are recommended to slow the emergence of anthelmintic resistance. Furthermore, meaningful research, surveys and epidemiological studies of anthelmintic resistance depends on the availability of sensitive, molecular tests to measure the trait.

Allele specific polymerase chain reaction (PCR) can detect genetic mutations both in both nematode populations and in individual worms. Sensitive markers for anthelmintic resistance could therefore be found if the mutations that cause anthelmintic resistance could be identified (Von Samson-Himmelstjerna, 2006). Unfortunately the widespread and high level of benzimidazole resistance in parasitic nematodes will inevitably limit the practical diagnostic value of any molecular tests for benzimidazole resistance to the nematode species generally of lesser importance that have yet to develop resistance. However, the elucidation of molecular markers for macrocyclic lactone resistance could still prove to be timely, allowing resistance to be identified at an early stage, before genetic changes in the parasite population reach the point where significant treatment failure occurs (Gilleard and Beech, 2007). Once molecular markers for anthelmintic resistance have been identified, then rapid, accurate, sensitive and relatively high

throughput PCR based methods, such as real time PCR or pyrosequencing (Von Samson-Himmelstjerma and Blackhall, 2005) could afford assays for practical use in the field to monitor changes in the population genetics of resistance.

1.6.3 Molecular mechanisms by which anthelmintic resistance might develop

In order to understand the mechanisms of anthelmintic resistance it is first necessary to understand the mechanisms of anthelmintic drug action. Anthelmintic drugs act on nematode parasites at a molecular level, by weakening drug binding to biochemical receptor sites, or by mimicking the action of biochemical transmitter substances on the receptors. For example, benzimidazole drugs bind to sites on tubulin molecules forming a tubulin-benzimidazole complex that prevents the polymerisation of α and β tubulin heterodimer subunit assemblies that make up microtubules (Lacey, 1988). The ganglion blocking drugs act as agonists at nicotinic acetylcholine receptors at the nematode neuromuscular junction causing a spastic paralysis (Sangster and others, 1998b). The actual actions of macrocyclic lactone anthelmintics at a molecular level are contentious, but are thought to involve GluCl and GABACl genes, or to be associated with overexpression of P-gps. Anthelmintic resistance therefore emerges when: (i) a target receptor becomes modified to reduce the binding affinity of the anthelmintic, so that the drug no longer recognises the target and is ineffective; (ii) the number of receptor sites is amplified, overcoming the action of the drug; (iii) pre-existing xenobiotic removal mechanisms are enhanced, preventing the drug from accessing its site of action; or (iv) mechanisms arise leading to enzymatic modification of the anthelmintic drug, leading to its inactivation or removal (Wolstenholme and others, 2004). One or more of these changes may be required before phenotypic manifestation of anthelmintic resistance arises.

1.6.3.1 Known genetic mutations causing anthelmintic resistance

1.6.3.1.1 Benzimidazole resistance

Benzimidazole resistance is associated with mutations in tubulin genes that prevent drug binding. Both α and β tubulin have many isoforms, coded by several genes (Geary and others, 1992). The isoforms that are present vary between different tissues of the same organism and between different helminth species (Lacey, 1988). Consequently, several different polymorphisms in β tubulin can be correlated with benzimidazole resistance (Prichard, 2001), and different polymorphisms may be important in different helminth species.

A single nucleotide polymorphism (SNP) mutation was identified in benzimidazole resistant *H. contortus* and *T. colubriformis* resulting in a single amino acid substitution from tyrosine to phenyalanine at position 200 (F200Y) of the polypeptide encoded by the isotype 1 β-tubulin gene (Kwa and others, 1994). The same point mutation also occurs in benzimidazole resistant *T. circumcincta* (Elard and others, 1996). This F200Y SNP is considered to be the most important mutation conferring benzimidazole resistance, for example the mutation has been shown to be functionally significant with respect to the benzimidazole resistance phenotype by transfection of the gene into *C. elegans* (Kwa and others, 1995). However, while polymorphism in the p200 codon of *T. circumcincta* is associated with survival of homozygous resistant genotypes, some heterozygous and homozygous susceptible genotypes also survive, implicating the involvement of other resistance mechanisms in the β tubulin isotype 1 gene (Silvestre and Cabaret, 2002), mutations in other tubulin genes (Lubega and others, 1994), or a role of non-tubulin resistance mechanisms. The substitution from tyrosine to phenylalanine at position 167 (F167Y) of the polypeptide encoded by the isotype 1 β-tubulin gene has been found in *T. circumcincta* and *H. contortus*, but not in *T. colubriformis* (Silvestre and Cabaret, 2002), further implying that the genetics of benzimidazole resistance may differ between nematode genera. The F167Y SNP has been identified in benzimidazole resistant *H. contortus* in the absence of the F200Y mutation (Prichard, 2001). A third isotype 1 β-tubulin mutation (E198A) has been found

in isolated populations of *H. contortus* in association with benzimidazole resistance, in the absence of F200Y or F167Y (Ghisi and others, 2007). Examination of the *H. contortus* genome sequence indicates that there are at least two β-tubulin genes in addition to the isotype 1 and 2 genes that have been studied to date (Gilleard, 2006).

Genetic pathways involving cell membrane efflux pumps (for example, P-gps) or drug metabolism and excretion (for example, cytochrome p450) are also implicated in benzimidazole resistance. The P-gps are ubiquitous, highly conserved transmembrane molecules, belonging to the ATP-binding cassette (ABC) protein family that mediate transport of lipophilic peptides across cell membranes. Fourteen P-gp genes have been described in *C. elegans* (Geerts and Gryseels, 2000) and seven have been found in *H. contortus* (Kerboeuf and others, 2003), although it seems likely that more will be present, and that mutations associated with anthelmintic resistance will not be easily found (Beugnet and others, 1997; Kerboeuf and others, 1999; Blackhall and others 2008). Cytochrome p450 is involved in the enzymatic oxidation of compounds including anthelmintic drugs and has been implicated in nematode anthelmintic resistance (Kotze, 1997; Kotze and others, 2006b). Cytochrome p450 has been shown to be a major mechanism involved with insecticide resistance (for example Kotze, 1993; Willoughby and others, 2007). Furthermore, it is likely that other hitherto undiscovered xenobiotic removal mechanisms may play a role in anthelmintic drug resistance.

In the case of the narrow spectrum, fasciolacidal benzimidazole anthelmintic, triclabendazole, it is thought that the active sulfoxide metabolite of the drug blocks parasite tegumental secretions. Triclabendazole resistance does not appear to involve the β-tubulin gene and it has been suggested resistance may be caused by enhanced metabolism of triclabendazole sulfoxide to the relatively inert sulfone metabolite (Robinson and others, 2004).

Thus, it appears that high levels of benzimidazole resistance involve mutations at several genetic loci, and that the relative significance of these mutations differs both between

parasitic nematode genera and between populations of the same species. Similar complexities undoubtedly exist in the molecular basis of resistance to the other anthelmintic groups.

1.6.3.1.2 Ganglion blocking agent resistance

Imidazothiazole and tetrahydropyrimidine resistant *H. contortus* have reduced binding affinity for the drug at nicotinic acetylcholine receptors (Sangster and others, 1998b), while in *C. elegans* levamisole resistance has been associated with an absence of one of two populations of nicotinic acetylcholine receptors at the neuromuscular junction (Richmond and Jorgensen, 1999). However, the molecular basis for differences between ganglion blocking agent sensitive and resistant nematodes remains obscure. Nematodes possess a large family of nicotinic acetylcholine receptor genes (Lewis and others, 1987). These genes have been studied in *C. elegans* and to date, polymorphisms associated with anthelmintic resistance have not been identified. The fact that different molecular mechanisms are responsible for ganglion blocking agent resistance in different genera (Waller and others, 1995), might imply that *C. elegans* is not a good model for the study of anthelmintic resistance in parasitic nematodes.

1.6.3.1.3 Macrocyclic lactone resistance

The macrocyclic lactone anthelmintics act on ligand gated channels (Dent and others, 2000) including glutamate (GluCl) (Blackhall and others, 1998b) and GABA-gated (GABACl) chloride channels (Blackhall and others, 2003). Glutamate and GABA are neurotransmitters that regulate muscle contractions, regulating activities such as feeding, locomotion and reproduction (Yates and others, 2003). However, the mechanisms responsible for the development of resistance are poorly understood. Variations in allele frequencies between resistant and susceptible *H. contortus* have been shown in a putative subunit GABACl gene (Prichard, 2001), but causal associations with ivermectin resistance have not been demonstrated. A functional SNP mutation has been shown at codon 256 (L256F) in a GluCl subunit gene of the cattle nematode parasite, *Cooperia oncophora*, associated with ivermectin resistance (Njue and others, 2004). However,

this mutation does not appear to be consistently present in ivermectin resistant *C. oncophora* and has yet to be found in any other parasitic nematodes. In addition to the ligand gated chloride channels, resistance mechanisms are also thought to involve a mutation in a P-gp gene (Blackhall and others, 1998a; Kerbouef and others, 2003), leading to more rapid drug clearance from the nematode parasite.

Relevant polymorphisms in candidate genes have not been consistently identified and resistance to ivermectin might depend on the accumulation of mutations in several genes. Furthermore, it is possible that different mechanisms will predominate in different isolates of macrocyclic resistant parasitic nematodes (Gill and Lacey, 1998). Some of these mutations that are involved with xenobiotic drug regulatory mechanisms may also be involved with multiple anthelmintic drug resistance.

In *H. contortus*, macrocyclic lactone resistance can be experimentally selected for by underdosing in just three nematode generations (Coles and others, 2005). In *T. circumcincta* in the field, macrocyclic lactone resistance has emerged rapidly after only two years use of ivermectin for suppressive nematode parasite control in the south-east of Scotland (Sargison and others, 2007a), and after just two treatments following a drought period in Western Australia (Sangster and Gill, 1999). The rapid emergence of macrocyclic lactone resistance may be because it is inherited as a dominant trait (Dobson and others, 1996; Coles, 1996), but also implies the pre-existence of mutations in unselected populations. The rapid emergence of macrocyclic lactone resistance is also consistent with a single major molecular mechanism, because multiple mutations developing simultaneously would intuitively be rare, unless they are genetically linked.

The results of reciprocal genetic crossing experiments between the ivermectin resistant CAVR strain and a susceptible isolate of *H. contortus* are consistent with a single locus conferring resistance in a genetically dominant fashion (Dobson and others, 1996; Le Jambre and others, 2000). However, the genetic basis of ivermectin resistance in the

CAVR isolate may be shown with time to differ from other *H. contortus* isolates and field populations.

1.6.3.1.4 Aminoacetonitrile derivatives

In the past, the introduction of every new class of anthelmintic drug has preceded basic knowledge of its mode of action and information required to gauge the risks of development of resistance. However, the mode of action of the AADs has been investigated by forward genetic screening of drug resistant mutants of *C. elegans* and *H. contortus* and seems to involve a unique nematode-specific clade of acetylcholine receptor subunits (Kaminsky and others, 2008). Should an AAD such as monepantel reach the market within the foreseeable future, it will almost certainly have been shown to be effective against nematode parasites that are resistant to the three existing broad spectrum anthelmintic groups. The AADs will therefore provide a short-term solution for the control of multiple drug resistant nematodes. However, it is possible that certain non-specific drug regulatory mechanisms conferring resistance to the AADs are already present in parasitic nematode populations at a higher level than they were before the introduction of the existing drugs, having been selected for by those drugs. The emergence of anthelmintic resistance to the AADs could therefore be rapid, underpinning the need for molecular markers to elucidate the population genetics of AAD resistance, providing a basis for advice about their responsible use.

1.6.3.1.5 Multiple anthelmintic drug resistance

All of the cases of macrocyclic lactone resistance in *T. circumcincta* that have been reported in the UK, have also involved resistance to benzimidazole and imidazothiazole anthelmintics (Sargison and others, 2001; Cheng and others, 2003; Sargison and others, 2007a). This might suggest that ivermectin treatment selected for subpopulations which already had inherently increased levels of resistance to benzimidazole and levamisole anthelmintics (Mottier, 2008). Alternatively, it might simply reflect the high prevalence of benzimidazole resistance. This situation could be explained by the linkage of alleles conferring resistance to benzimidazole, imidazothiazole and macrocyclic lactone

anthelmintics, arising due to the close proximity of the alleles on the same chromosome. Alternatively, multiple anthelmintic drug resistance may arise due to a shared mechanism, such as the up-regulation of drug regulatory mechanisms. It is also possible that different selection pressures in different flocks may have led to different molecular bases of multiple anthelmintic drug resistance (Van Wyk and others, 1997a).

1.7 Methods for finding molecular markers for anthelmintic resistance

1.7.1 Candidate gene studies

To date, most of the research aimed at identifying molecular markers for anthelmintic resistance has focussed on the investigation of possible associations between candidate genes and the resistance phenotype.

1.7.1.1 Identification of candidate genes

The conventional approach to identifying molecular markers for anthelmintic resistance predominantly involves first selecting potential candidate genes on the basis of knowledge of the anthelmintic drug's mode of action. Within this context, a gene is considered to be an open reading frame of DNA, defined by start and stop codons, containing the information for a single specific polypeptide. The presence of introns means that genes can be considerably longer than the actual coding sequence, sometimes being several hundred base pairs (bps) in length. Therefore, the study of a single gene is in itself a considerable task. Genes encoding proteins that are involved in the biological effects of and physiological responses to the drug become candidates for analysis. Some such potential *H. contortus* candidate genes can be identified by gene expression or complementary DNA probe studies using standard molecular biological methods. However, in the absence of an assembled, annotated *H. contortus* genome, many genes

of interest must be identified using bioinformatics, searching for homology with known *C. elegans* genes.

Once SNP or indel (insertion or deletion) mutations have been identified within candidate genes, it is necessary to investigate genetic and biochemical differences between susceptible and resistant parasites, in order to obtain circumstantial evidence for the pharmacological relevance of altered proteins in conferring resistance. Studies are then required to compare levels of their transcription between resistant and susceptible nematodes (Gilleard and Beech, 2007), for example using a microarray to look for evidence of their upregulation, or quantitative real time PCR to demonstrate higher levels of the relevant mRNA in the resistant nematodes. Functional studies of the effect of the mutation on drug binding, for example when expressed in *Xenopus* oocytes, or the effect on sensitivity to the drug when the mutation is expressed in *C. elegans* can then be used to prove a causal relationship between a mutation in a candidate gene and the resistance phenotype.

1.7.1.2 Demonstration of association between candidate genes and resistance phenotype

The standard approach to demonstrating an association between mutations in candidate genes and phenotypic expression of resistance is to compare parasite populations that differ in their response to treatment. However, the extreme genetic variation that exists in *H. contortus* populations (Troell and others, 2006; Redman and others, 2008) means that genetic differences exist between the two isolates, regardless of any association with resistance. This problem can be surmounted by deriving susceptible and resistant lines from a single isolate by experimental selection for anthelmintic resistance. This can be achieved *in vivo*, by underdosing successive nematode generations in sheep (for example Coles and others, 2005), or rodents (for example, Molento and others, 1999), and *in vitro* by selecting larvae that hatch, feed or develop in discriminatory concentrations of anthelmintic drug over a period of several generations (for example, Egerton and others, 1988; Echevarria and others, 1993; Rohrer and others, 1994), to produce significant

phenotypic differences between treated and untreated groups. Since these lines share an initial genetic pool, then differences between selected resistant and parent susceptible lines can be attributed to anthelmintic resistance.

A different approach that concurrently identifies candidate genes and demonstrates their role in the phenotypic expression of resistance involves the comparison of specific regions of the genome that might be associated with the mechanisms of drug action between field selected anthelmintic susceptible and resistant nematode populations. This approach was used for the identification of the GluCl subunit gene L256F SNP, associated with ivermectin resistance in *C. oncophora* (Njue and Prichard, 2004).

1.7.1.2.1 Potential problems arising from experimental associations between candidate genes and resistance phenotype

The value of comparison of phenotypically different nematode populations, derived by experimental selection for anthelmintic resistance, can be limited by the degree of genetic diversity within the parent nematode population and how freely genetically distinct subpopulations of nematodes interbreed. For example, if the parent population consists of an admixture of distinct subpopulations, as has been demonstrated in a French isolate of *T. circumcincta* using a panel of microsatellite markers (Grillo and others 2006), then it is possible that selection pressure for resistance may favour a specific subpopulation that is initially more resistant. In this case, the final genetic analysis may reveal differences between the initial subpopulations that are related to the genetic diversity of the parent population, but not to the selected resistance mechanism. Genetic diversity in the parent population can also confound the results if population bottlenecks occur during the selection process, whereby only one subpopulation survives (Gilleard and Beech, 2007).

A potential disadvantage of deriving susceptible and resistant lines from a single isolate, by experimental selection for anthelmintic resistance, is that these approaches may select for characteristics of general fitness, rather than solely for anthelmintic resistance. For

example, if eggs are collected soon after the prepatent period, then the experiment may select for short prepatent period rather than resistance, or larvae that hatch, feed, or develop in *in vitro* pressurisation experiments may do so because they are inherently fitter, rather than because they are inherently resistant. This could lead to selection for loci conferring fitness traits in addition to and independently of anthelmintic resistance phenotype (Gilleard and Beech, 2007).

A further problem is that experimental selection for anthelmintic resistance by *in vitro* pressurisation, or *in vivo* by underdosing may not involve the same mechanisms as field selection. For example, field selection for anthelmintic resistance occurs when parasitic nematode stages are exposed to a full dose of anthelmintic, while *in vitro* selection involves exposure of free living stages to anthelmintic concentrations that would not confer phenotypic resistance in the field.

The major problem with the comparative genomic approach is that differences between populations are as likely to be associated with other characteristics that define the different populations as they are to be involved with anthelmintic resistance phenotype.

1.7.1.2 General limitations of candidate gene approaches

While conventional candidate gene studies are certainly worthwhile, they are constrained by difficulties with providing proof of associations between mutations in genes of interest and phenotypic anthelmintic resistance. Furthermore, association studies of candidate genes cannot directly demonstrate that a particular mutation is capable of conferring anthelmintic resistance, because it is possible that a polymorphism in a genetically-linked gene could be responsible for the resistance. Candidate gene studies may also fail if the true major resistance mutation is in a gene that has not been anticipated based on knowledge of the molecule.

To date, candidate gene approaches have not been particularly rewarding for the identification of a major single molecular marker for macrocyclic lactone resistance.

This may simply reflect the fact that the mechanism of macrocyclic lactone resistance is complex and does not involve a single major determinant. Furthermore, several genes may be involved with known drug actions or metabolism, for example at least seven P-gp genes have been found in *H. contortus* (Kerboeuf and others, 2003), and it is possible that the relevant genes have yet to be studied. Alternatively, failure to identify a major single molecular marker may be due to the involvement of genes that have hitherto not been found or recognised as being associated with drug action. For example, consideration of candidate genes solely as regions coding for proteins or polypeptides, ignores regions of DNA that code for ribosomal and transfer RNA that could be important for cell viability when exposed to anthelmintic drugs, or regions that act as binding sites for regulatory proteins that are important in gene regulation. These considerations illustrate the fundamental limitations of conventional candidate gene approaches.

1.7.2 Genome-wide approaches to identify anthelmintic resistance loci

An alternative approach to the identification of molecular markers is to exploit the *H. contortus* genome to enable genome wide comparison between field-selected susceptible and resistant isolates. A major advantage of this approach is that it does not rely on pre-conceived ideas about putative resistance genes of interest and potentially identifies genes conferring anthelmintic resistance through gene regulatory mechanisms, non-specific xenobiotic defence mechanisms and known sites of drug action. Similar genetic and functional genomic approaches have been successfully used to identify a transposon insertion mutation in a cytochrome p450 gene as a major determinant of dichloro-diphenyl-trichloro-ethane (DDT) and multi drug resistance in *Drosophila melanogaster* (Daborn and others, 2002).

7.1.2.1 Exploitation of the H. contortus *genome*

About 600 megabases of shotgun genomic DNA sequence has now been generated by the Wellcome Trust Sanger Institute *H. contortus* genome sequencing and mapping project (J. Gilleard, *personal communication*), representing about 6 fold genome coverage. Based on examination of shotgun sequence, it is estimated that two sequences of DNA from different individual *H. contortus* haplotypes typically differ at between 20 and 30 nucleotide positions per 1000 bp (kb) (Gilleard and Beech, 2007). This sequence diversity is due to a combination of SNPs, insertions and deletions, which could enable the use of bioinformatics to generate a large panel of genome-wide, polymorphic, neutral molecular markers. Molecular markers can be exploited to identify genes that might be associated with anthelmintic resistance by multilocus genotyping and genetic linkage analysis. Thus, the availability of 6 fold *H. contortus* genomic sequence provides a timely opportunity for the development of genome-wide approaches to study macrocyclic lactone anthelmintic resistance in *H. contortus*.

7.1.2.2 Microsatellite markers

Microsatellites are highly polymorphic sequences of 2 or 3 nucleotide pair tandem repeats, usually less than 150 bp long, scattered throughout the genome. The most abundant dinucleotide repeats are cytosine-adenine and guanine-adenine. Variation in the number of repeats present is thought to originate from slippage events during DNA replication and mismatch repair, giving rise to polymorphism. Microsatellites are found in both coding and non-coding sequence throughout the genome and are considered to be neutral markers (Bennett, 2000; Tautz, 1989; Zane and others, 2002). The mutation rate in microsatellites is much greater than simple base substitution rates, so microsatellite markers are more abundant than alternative neutral SNP markers. Once flanking sequence is known, then microsatellites can be amplified by PCR from small amounts of DNA, followed by relatively straightforward assignment of alleles based on their size. Microsatellite markers are widely used in this way for DNA fingerprinting.

In the absence of significant amounts of genome sequence, microsatellites are generally found by screening small insert genomic libraries using oligonucleotide probes, or by using primers designed to flank microsatellites which have already been identified in closely related species. However, parasitic nematodes and *C. elegans* are phylogenetically distant from each other and few orthologous microsatellites have been found (John Gilleard, *personal communication*). The availability of significant amounts of *H. contortus* genome sequence, therefore, affords a timely opportunity to use bioinformatics to identify potentially large numbers of useful, polymorphic microsatellites from which to choose population genetic markers. Some have been published (Hoekstra and others, 1997a; Otsen and others, 2000a; Otsen and others, 2000b; Otsen and others, 2001). Numerous further potential microsatellites have since been identified by applying bioinformatics to shotgun sequence available from the *H. contortus* genome project. Many of these microsatellites have proven to be polymorphic and the prospect of a useful large panel of markers now exists (Redman and others, 2008; J. Gilleard, *personal communication*).

If genes conferring anthelmintic resistance are situated close to a neutral microsatellite marker on the same chromosome, then genetic linkage will give rise to increased frequency of a particular microsatellite allele in resistant nematodes relative to susceptible nematodes. Therefore, if the position of microsatellite markers on the genome is known, genes conferring the anthelmintic resistance phenotype could be identified searching for microsatellite polymorphisms linked to the resistant phenotype between resistant and susceptible nematodes, and multi-locus microsatellite linkage analysis. This process would require a large panel of at least 100 useful polymorphic microsatellites, evenly distributed across the genome, and a fully annotated genome sequence. While neither of these tools currently exists, there is a realistic prospect that they will become available in the foreseeable future, enabling a genome wide approach to identify genes conferring macrocyclic lactone resistance in *H. contortus*. Once new candidate genes have been identified, then resistance conferring mutations which could

afford molecular markers could be identified using conventional molecular biological methods.

1.7.2.3 Genetic crossing experiments

1.7.2.3.1 Genetic crosses between resistant and susceptible nematodes

Genetic crossing of parasitic nematodes can be achieved by the surgical transfer of female nematodes from one strain and male nematodes from another into the abomasum of a recipient host (Le Jambre, 1977; Le Jambre, 1979; Le Jambre and others, 1979; Le Jambre, 1981; Sangster and others, 1998a; Le Jambre and others, 1999b; Le Jambre and others, 2000; Le Jambre and others, 2005). In these experiments, F_1 progeny have been assessed for their resistance phenotype by *in vitro* or *in vivo* assays and then used to orally infect sheep to produce F_2 generations from which the degree of dominance of the trait can be assessed.

A possible genetic approach to identify candidate genes and concurrently demonstrate an association between mutations and the field resistance phenotype would be to perform a genetic cross between resistant and susceptible field isolates. Taking the conceptually simplest example of resistance being conferred by a single autosomal recessive mutation, if the female parents are homozygous for alleles conferring resistance (*rr*) and the male parents are homozygous for alleles conferring susceptibility (*ss*), then the F_1 generation will all be heterozygous (*rs*). If a genetic cross is performed between these parents and the F_1 progeny allowed to interbreed, the genotype frequencies in the F_2 population should be 1 (*rr*): 2 (*rs*): 1 (*ss*). Hence if the genotypes of individual F_2 larvae are phenotypically resistant to the drug, then 100% will be *rr* for the resistance allele (or for a genetic marker tightly linked to that allele). In contrast, for unlinked genetic markers, the different alleles from the two parental backgrounds will be present at equal frequency. Thus, if the resistance phenotype of individual F_2 nematodes can be reliably determined, then determining the relative frequency of the respective parental alleles in resistant F_2 larvae for a large number of neutral genetic markers can be used to determine whether each marker shows linkage to the resistance-conferring mutation.

The details are different for dominant, semi-dominant or multigenic traits but the basic concept is the same.

This approach overcomes potential problems caused by selection for fitness rather than anthelmintic resistance, selection for anthelmintic resistance mechanisms that are not significant in the field, and identification of markers for traits that merely characterise differences between isolates. Genetic crossing experiments in parasitic nematodes typically involve mass matings of up to 100 male and 100 female nematodes. However, one potential confounding factor for the approach is the high level of genetic diversity in the parental populations. This may complicate the determination of the parental origin of alleles detected in F_2 progeny (whether they are derived from the resistant or susceptible parental isolate). This problem could be overcome in two ways. Either the parental lines could be inbred to reduce within population genetic diversity while maintaining between population diversity before performing mass matings, or alternatively genetic crosses could be performed by single pair matings.

Another confounding factor for the genetic crossing approach described above is that it requires the ability to accurately determine the resistance phenotype of the individual worms. However this is not straightforward, for example not all eggs that fail to hatch, or larvae that fail to feed or develop in bioassays are susceptible to anthelmintic treatment, and there are no straightforward, non-invasive means of identifying susceptible nematodes *in vivo*. Assigning an accurate phenotype to individual F_2 nematodes presents a major challenge to the genetic crossing approach outlined above.

1.7.2.3.1 Genetic backcross experiments

Backcrosses between the F_1 progeny of genetic crosses and parental strains have been performed (Le Jambre and others, 1979; Le Jambre and Royal, 1980; Le Jambre and others, 1999b) to determine whether resistance appears to be conferred by a single locus.

An alternative approach to identify molecular markers that are genetically linked to the region of the genome in which a major mutation conferring resistance resides would be to repeatably backcross a resistant *H. contortus* isolate against a known susceptible isolate. This could be achieved by first making a genetic cross between the two isolates, exposing the progeny to anthelmintic, then backcrossing the resistant progeny with the susceptible parent isolate. After a number of such backcrosses, the genetic background of the progeny population should essentially be the same as the original susceptible parent population, except for regions surrounding (linked to) the resistance conferring mutation. Therefore, for unlinked genetic makrers, the alleles (and their respective frequencies) present in backcrossed population should be the same as the susceptible parental populations. However for markers linked to the mutation conferring resistance, the alleles (and frequencies) should correspond to those found in the resistant parental populations. A prerequisite of this approach is that parental populations need to be sufficiently genetically divergent to enable maternal and paternal alleles to be distinguished in the backcrossed population.

This backcrossing approach would overcome the need to make genetic crosses of individual nematodes and to accurately determine the resistance phenotype of individual nematodes, because the analysis is based on comparative genomics of mass nematode populations.

1.7.2.4 Linkage analysis

Offspring inherit a single copy of each chromosome from each parent. The process of meiotic recombination causes segments of DNA to be exchanged between homologous chromosome pairs. Genes or neutral molecular markers that are present on different chromosomes segregate independently during meiosis and are then distributed independently amongst progeny. Likewise, some genes that are on the same chromosomes may segregate independently due to genetic crossover during meiosis and end up on different chromosomes in the offspring. Genes that are close together on the same chromosome are more likely to remain associated during meiosis than genes that

are far apart, and are said to be linked. Thus, any neutral molecular marker that is selected for by genetic crossing experiments between anthelmintic resistant and susceptible nematodes is likely to be linked to a major gene conferring resistance. The degree of linkage is negatively correlated with distance from the gene of interest, providing a method of mapping that gene relative to the markers. Identification of the gene of interest therefore requires a large panel of polymorphic molecular markers with which to characterise parental strains, and knowledge of the genomic location of the markers.

Once a fully assembled and annotated *H. contortus* genome is available, the position of molecular markers will become known. Unfortunately, the 6-fold *H. contortus* shotgun genomic sequence that is currently available cannot be assembled due to a high level of polymorphism within the MHco3 (ISE) strain that has been used for the project (John Gilleard, *personal communication*). In the absence of information about the position of microsatellites on the physical map of the *H. contortus* genome, genetic crossing experiments to identify major genes conferring anthelmintic resistance are, therefore, ahead of their time.

1.7.2.4.1 Inbreeding of the MHco3 (ISE) strain of H. contortus

The MHco3 (ISE) strain of *H. contortus* was inbred (I) from a highly polymorphic Moredun susceptible (SE) population (Otsen and others, 2000b; Otsen and others, 2001) by infecting sheep with the L_3 progeny from 15 generations of single female nematodes (Roos and others, 2004). The MHco3 (ISE) strain was chosen for the genome project because it was considered to be less polymorphic than other characterised, anthelmintic susceptible strains. However, the MHco3 (ISE) strain produced in this manner remained highly polymorphic, because the single female *H. contortus* nematodes used during each step would have contained the progeny of several males (Redman and others, *In Press*). A potential solution to expedite the process of *H. contortus* genome assembly would, therefore, be to develop an inbred MHco3 (ISE) line from the progeny of a mating

between single female and male parents. Such inbred lines would also be very useful for genetic crossing and mapping experiements.

1.7.2.4.2 Linkage analysis to create a genetic map

Genes that are close together on the same chromosome do not segregate independently during meiosis, but may remain associated and may be inherited together. Measuring the degree of linkage provides a way of mapping genes. In the absence of an annotated genome, the position of microsatellite markers relative to each other can be determined by the creation of a genetic map. A genetic map for microsatellite markers, using the *H. contortus* genome project MHco3 (ISE) strain, could be produced by performing a genetic cross between two parent lines and then interbreeding the progeny to produce an F_2 generation. About 100 individual F_2 nematodes could then be genotyped for a panel of microsatellite markers. Those that are closely linked on the same chromosome would tend to associate with each other in the F_2 generation, while those that are not linked would associate randomly. The magnitude of the association would be negatively correlated with the size of the distance separating the markers, and could therefore be used to create a genetic map. The parent populations would need to be genetically different, so that alleles could be distinguished in the progeny. This could be achieved using a single parent genetic crossing method on the MHco3 (ISE) strain *H. contortus*, to produce divergent, inbred lines.

1.7.3 Biology of parasitic nematodes

The design and interpretation of genetic crossing experiments, and the application of molecular markers to study of the population genetics necessitates a sound understanding of basic parasitic nematode biology and genetics. Current understanding of parasite reproduction in response to different conditions is limited. For example, knowledge of how many males mate with single female nematodes, the age or stage at which mating first occurs, the period for which female nematodes shed fertilised eggs

after mating and the number of eggs shed by female nematodes is a prerequisite for the design of genetic crossing studies. The analysis of genetic crossing experiments is based on an assumption that parasitic nematodes are dioecious, sexually reproducing, diploid organisms, with genetic recombination being a requirement of meiosis. However, if some are hermaphroditic, or sexual meiotic recombination does not occur, then the interpretation of genetic crossing experiments will be difficult.

1.8 Aims of study

In summary, control of nematode parasites is a prerequisite for efficient, economic sheep production. Efficient nematode parasite control in intensive production systems depends on the use of effective anthelmintic drugs, and is therefore threatened by the emergence of multiple anthelmintic drug resistance. The prospect of a new class of anthelmintic drug is unlikely to provide a long term solution, because it is likely that non-specific drug regulatory mechanisms involved with anthelmintic resistance are already present in parasitic nematodes. It ought to be possible to manage nematode parasites of sheep in a manner that minimises the impact of anthelmintic resistance and some recommendations, aimed at ensuring maximum drug efficacy are straightforward. However, recommendations aimed at ensuring that only a small proportion of the total nematode population is exposed to anthelmintic treatment are based on mathematical modeling and are unproven, due to a lack of understanding of the population genetics of resistant nematodes. It is unclear whether or not the use of macrocyclic lactone drugs as systemic endectocides increases the risk of anthelmintic resistance. If they do, then the development of alternative strategies for the control of sheep scab is essential. Sensitive, accurate molecular markers for anthelmintic resistance are therefore needed, in order to elucidate the population genetics of resistant nematodes. The pressing need to validate these recommendations, that are largely based on theoretical considerations, is demonstrated by the confirmation of a high prevalence of multiple anthelmintic drug

resistance in *Teladorsagia circumcincta* both in the south-east of Scotland (Sargison and others, 2007a) and elsewhere in the UK (Veterinary Laboratories Agency, 2008).

While they are worthwhile, conventional candidate gene studies may prove to be unsuccessful in the identification of major molecular markers for anthelmintic resistance. *H. contortus* is a convenient experimental model parasite and the prospect of a complete, annotated genome provides a timely opportunity for the development of genome wide, genetic crossing approaches to search for major molecular markers for anthelmintic resistance. The overall aims of this study were to develop such approaches. In the absence of large panels of mapped polymorphic genetic markers and a fully assembled and annotated genome, in some respects genetic crossing experiments are ahead of their time. However, this study aims to develop concepts and methodologies to be used in future mapping studies and, importantly, archived material from this study will provide a long-term valuable resource for ongoing and repeated analysis as molecular technologies and genomic resources for *H. contortus* are developed.

The study brings together experience of farm animal veterinary practice and clinical investigations of nematode parasitism in sheep, laboratory diagnostic parasitology and genomics. Standard gross and molecular parasitological methods are used: firstly to study aspects of the biology of *H. contortus* that are prerequisites for the achievement of a successful genetic cross and will underpin understanding of the relevance of any molecular markers that might be identified using this approach; secondly in the proof of concept of producing inbred lines of the MHco3 (ISE) isolate of *H. contortus* used in the genome project; and finally in backcrossing regions of the genome linked to ivermectin resistance conferring polymorphisms from resistant isolates into the genetic background of the MHco3 (ISE) isolate.

Chapter 2: General materials and methods

2.1 Gross parasitological methods

2.1.1 The diagnosis of parasitic gastroenteritis

The list of common causes of ill thrift in lambs includes poor nutrition, previous perinatal disease, parasitic gastroenteritis, cobalt and selenium deficiencies, fascioliasis, respiratory disease, lameness and sheep scab. The diagnosis of parasitic gastroenteritis as a cause of ill thrift therefore requires a rational scientific approach, involving a focused disease history (including information about anthelmintic treatments and pasture management for the control of nematode parasites), clinical examination of the flock and appropriate sample collection (Sargison, 2008). Frequently, parasitic gastroenteritis occurs alongside other potential causes of ill thrift, emphasising the importance of a rational diagnostic approach to determine which are primary causes and which are incidental or consequential.

2.1.1.1 Clinical examination

This course of study was conceived because of difficulties that arose concerning the management of parasitic gastroenteritis in sheep flocks in the south-east of Scotland. While some disease outbreaks were caused by a single predominant parasite species, in many cases the clinical manifestation of parasitic gastroenteritis was confused by the presence of more than one nematode parasite species, resulting in non-specific clinical signs of diarrhoea, loss of appetite, weight loss and death.

2.1.1.1.1 Teladorsagia circumcincta

Teladorsagiosis causes hyperplastic inflammation of the abomasal mucosa, replacement of gastric secretory cells by unfunctional cells, hypoproteinaemia due to leakage across the damaged mucosa, and impaired abomasal function associated with alteration of its

pH. Clinical disease is typically, but not always, seen in lambs during the summer, characterised by watery diarrhoea, loss of appetite and weight loss (for example, Sargison and others, 2002).

2.1.1.1.2 Trichostrongylus vitrinus and *Trichostrongylus colubriformis*

Trichostrongylosis causes mucosal hypertrophy and villous atrophy of the proximal small intestine, leading to protein leakage, electrolyte and mineral imbalance. Disease is typically seen during autumn and early winter months as immunity to *T. circumcincta* becomes established, and is characterised by anorexia, dark coloured scour, poor skeletal growth, poor wool quality and chronic ill thrift (for example, Sargison, 2000a).

2.1.1.1.3 Nematodirus battus

Nematodirosis causes catarrhal inflammation of the small intestine, typically leading to severe disease during May and June in young lambs between 6 and 12 weeks-old. Nematodirosis is characterised by acute onset profuse diarrhoea, lethargy, abdominal pain, rapid weight loss, dehydration and death (for example, Sargison, 2006c).

2.1.1.1.4 Haemonchus contortus

H. contortus feed on blood and cause anaemia and hypoproteinaemia. In endemically affected regions, haemonchosis is usually seen in lambs during late summer, but can also occur in naïve animals at any time of year. Haemonchosis is characterised by anaemia, submandibular oedema, ascites, ill thrift and death. Scour is seldom present when *H. contortus* is the major nematode parasite present (for example, Sargison and others, 2007b).

2.1.1.2 Postmortem examination

2.1.1.2.1 Gross findings

When the abomasa of freshly dead sheep were opened without allowing escape of digesta, heavy parasite burdens were assessed from the presence of swirling movement of the surface of the digesta. Adult *H. contortus* on the mucosal surface of the

abomasum were easily visible as 2 to 3 cm long nematodes. Female nematodes had a characteristic 'barbers pole' appearance, where the blood filled gut spiraled around the uterus. *T. circumcincta* were also visible on the abomasal mucosa as up to 1 cm long, slender, hair-like and brown-coloured nematodes. When required, the small intestine was ligated at its proximal end and removed by cutting down through the pancreas. *T. vitrinus* are small and hair-like, less than 7 mm long and were only just visible to the naked eye. *N. battus* were seen as slender 2 cm long white worms in the lumen of the intestine. *N. battus* were sometimes seen in tangled knots with a characteristic 'cotton wool ball' appearance but in some animals which died as a result of acute nematodirosis, many of the worms had been expelled and few were found in the small intestine. Other gross postmortem signs such as thickening of the abomasal mucosa and glandular hypertrophy, abnormal intestinal contents, catarrhal enteritis, pallor, or ascites were used to further support a diagnosis of gastrointestinal parasitism.

Histological examination of stained sections of abomasal or intestinal wall can provide diagnostic information, but was not considered to be necessary for this study. Plasma pepsinogen concentrations, which are sometimes useful in cattle, are of limited diagnostic value in sheep.

2.1.1.2.2 Estimation of nematode worm burdens

Total nematode counts used in this study involved the postmortem collection of the abomasal or intestinal contents and mucosal washings into 0.85% saline (made up by adding 8.5 g of NaCl to 1 l of tapwater). The digesta were poured through a 500 μm sieve, to trap nematodes, while removing small particles and opaque dissolved substances. The nematodes and digesta retained in the sieve were then made up to 5 litres in 0.85% saline and thoroughly mixed while removing two separate 250 ml aliquots. The total nematode worm count was estimated by counting all of the nematodes present in this 10% subsample (or in a 100 ml, 2% sub sample when counts were high). When appropriate, numbers of adult male, adult female and immature larval stages of different nematode species were recorded separately. Numbers of arrested EL_4

were estimated by first soaking the washed abomasa in 0.85% saline at 37°C for about 6 hours, before stripping the mucosa into a known volume of 0.85% saline, mixing and withdrawing 10% aliquots.

Whenever nematodes could not be counted on the same day that they were recovered, 20 ml of 50% formalin was added to the 10% subsamples to allow for storage in 2% formalin and counting at a later date. Counting was then aided by first staining the preserved subsamples with about 2 ml of helminthological iodine (250 g potassium iodide and 50 g iodine made up to 500 ml with distilled water), before washing them over a 38 μm sieve and re-suspending in 500 ml of tapwater.

2.1.1.2.3 Identification of adult nematodes

Adult *H. contortus* in this study were identified by their relatively large (2 to 3 cm long) size and characteristic morphology. Adult male *H. contortus* nematodes were identified by their size and by the morphology of their spicules and bursa (Taylor and others, 2007a).

2.1.1.3 Ancillary laboratory tests

2.1.1.3.1 Faecal nematode worm egg counts (FWECs)

FWECs provide a reasonable estimate of the sheep host's nematode burden, enabling prompt diagnosis and treatment of parasitic gastroenteritis and providing a basis for effective and sustainable nematode control strategies for individual flocks. FWECs need to be based on freshly voided samples from at least 10 animals, to allow for the variation that occurs within groups of sheep. Bulked samples from 10 or more animals can provide useful information, but the results are harder to interpret than those from individual samples, and less suitable for experimental studies. There are limitations to FWECs, which were taken into account when interpreting results. Faecal nematode egg excretion can be altered for a number of reasons, such as variation in egg production between genera; sheep breed, age and reproductive status (Salisbury and Arundel, 1970); the length of time that the sheep have been off pasture; and the dry matter content of the

faeces. FWECs only indicate the presence of adult worms, but loss of production can occur when only larvae are present.

Field faecal samples were collected within two hours of removal of sheep from pasture to overcome some of the variation in FWECs associated with faecal concentration due to the effect of yarding on faecal dry matter. Faecal larval cultures were used to mitigate against the effect of variation in egg production between nematode genera.

Various methods can be used for faecal nematode egg counting, all involving the same basic principle, that the faecal mass is first broken up, and the eggs in a known quantity of faeces are then floated onto the underside of a counting chamber using saturated saline solution. This study employed a standard modified McMaster method (Large Animal Practice clinical investigations) and a cuvette method (Moredun Research Institute experimental studies).

2.1.1.3.1.1 Modified McMaster method

The modified McMaster method (Ministry of Agriculture, Fisheries and Food, 1986) does not rely on expensive laboratory equipment and is ideally suited to veterinary practice diagnostic laboratories. However, it is cumbersome when large numbers of samples must be examined, and the sensitivity of 50 eggs per gram (epg) is insufficient for some experimental purposes.

A small bowl and 'tea straining' (about 500 μm mesh) sieve were placed on a balance. Two grams of faeces were weighed into the sieve and 28 ml of saturated NaCl added. The faeces and saturated NaCl within the sieve were mixed with a spatula to break up the faecal matter. The coarse fibrous retentate was then discarded and the faecal suspension then agitated while removing an aliquot with a pipette. This was used to flood one chamber McMaster counting slide, before the pipette was reloaded and the procedure repeated to flood the second chamber. Loaded McMaster slides were left to stand for a few minutes before counting each trichostrongyle egg within the 10 lane

counting grids in both chambers (glass slides used in the Large Animal Practice have 10 lanes, while Perspex slides used elsewhere have 6 lanes). Using this protocol, each egg seen within the counting grids on the McMaster slide represented 50 eggs per gram.

2.1.1.3.1.2 Cuvette method

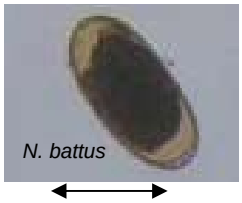
The cuvette method, developed by the Moredun Research Institute (Christie and Jackson, 1982) is straightforward, relatively quick and highly sensitive, but depends on the availability of dedicated laboratory equipment.

Faecal samples were collected into plastic bags and weighed. 10 ml of tapwater was added for each gram of faeces. The sample was thoroughly dispersed into and mixed with the water using a stomacher, before 10 ml was withdrawn and transferred into a 'tea straining' (about 500 μm mesh) sieve, placed over a small plastic bowl. Contents of the sieve were then flushed into the bowl using about 5 ml of tapwater, before discarding the coarse fibrous retentate. The filtrate was transferred into a flexible polyacrylamide centrifuge tube and centrifuged at 203 g (1000 rpm) for 2 minutes. The supernatant was then withdrawn using a vacuum line, before the pellet, containing trichostrongyle eggs, was re-suspended in saturated NaCl, gently mixed, and spun again at 203 g (1000 rpm) for 2 minutes. The flexible centrifuge tube was then clamped using artery forceps just below the meniscus, before transferring the contents above the clamp into a cuvette counting chamber. The cuvette was filled with saturated NaCl, sealed and left to stand for a few minutes, before counting trichostrongyle eggs on the underside of the upper face. Using this protocol, each egg within the cuvette represented 1 epg. Counting was simplified by counting eggs seen within a calibrated 3x or 9x eyepiece graticule, traversed in both directions across the cuvette (Bartley, 2007).

2.1.1.3.2 Identification of nematode eggs to genus or species level

The eggs of various different sheep gastrointestinal parasite genera, such as trichostrongyle eggs, strongyloid eggs, rhabditoid eggs, trichuroid eggs, anoplocephalid eggs and coccidian oocysts can be identified using these saturated saline floatation

methods, and it is important to differentiate those which have the potential to cause disease from those which are likely to be clinically irrelevant (Table 2.1). Other material such as mite eggs, fungal spores and pollen grains must also be differentiated from trichostrongyle nematode eggs.

Trichostrongyle eggs  100 µm  *N. battus* 100 µm	• Trichostrongyle eggs belonging to *Teladorsagia*, *Trichostrongylus, Haemonchus* and *Cooperia* genera are thin walled, oval shaped and between 70 and 100 µm long. The eggs may contain a morula of blastomeres or a pre-hatch larva, depending on the age of the faecal sample and their stage of development. While there are subtle differences in shape and size of some of the trichostrongyle eggs, for example those of *H. contortus* are smaller, while those of *T. colubriformis* are slightly flattened, giving the curvature of their walls an asymmetrical appearance; these differences cannot be reliably differentiated on gross microscopic examination. Trichostrongyle eggs of different genera can be stained with fluorescent agglutinins, which specifically bind to lectins on their surface. For example, *Haemonchus* spp. eggs can be identified using a fluorescein-labeled peanut agglutinin. However, this method is not inexpensive and requires access to a specialist fluorescent microscope. • *Nematodirus* spp.eggs are larger than the other trichostrongyle eggs, being 150 to 200 µm long, with a small number of discrete, large, round blastomere cells. *N. battus* has a dark, parallel-sided shell, while *N. filicollis* and *N. spathiger,* which do not have the same critical hatching requirements as *N. battus* and are of lesser clinical significance, have clearer shells with curved sides. The absence of *N. battus* eggs in faecal samples does not exclude a diagnosis of nematodirosis, because disease can occur during the pre-patent period of infection.
Strongyloid eggs  100 µm	• The eggs of *Chabertia* and *Oesophagostomum* (large intestinal strongyles) appear similar to those of the trichostrongyle nematodes, but are slightly larger and rounder. These strongyloid nematodes are seldom clinically significant in UK sheep, and most pathology occurs during their 6 to 8 weeks pre-patent period. At certain times of year, strongyloid eggs may contribute significantly to the FWECs of some classes of sheep without associated disease or ill thrift (for example, coproculture of ewe faeces during the autumn and winter months often yields predominantly *Chabertia* or *Oesophagostomum* spp. L_3). The presence of strongyloid eggs should be considered when interpreting FWECs of groups of sheep which have not recently been treated with an anthelmintic. • *Bunostomum* (hookworm) eggs are about 150 µm long with rounded ends and sticky shells to which faecal debris adheres. Hookworms were once common, but are now seldom identified in the UK.

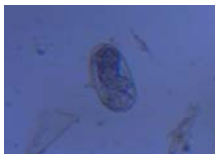
Rhabditoid eggs  100 µm	➧ *Strongyloides* spp. eggs are small, 40 to 60 µm long, thin walled and almost rectangular-shaped, containing a coiled L_1. The identification of these eggs is usually clinically insignificant, even when they are present in large numbers. *Strongyloides* spp. nematodes can have both parasitic and free-living lifecycles. Parasitic female L_3 penetrate the skin of their host then migrate through various organs to the small intestine, where unfertilised larvated eggs are produced by parthenogenesis. Free living lifecycles, with production of fertilised eggs occurs on the ground, with females sometimes laying eggs directly onto faeces. Thus, high *Strongyloides* spp. egg counts are sometimes identified in faecal samples collected off the ground. *S. papillosus* has a short pre-patent period, so eggs are often present in faecal samples collected between 10 and 14 days after anthelmintic treatment, and must be differentiated from trichostrongylid eggs to avoid the incorrect diagnosis of anthelmintic resistance.
Trichuroid eggs  100 µm	➧ *Trichuris ovis* (whipworms) are sometimes identified as incidental postmortem findings in sheep which have not been treated with an anthelmintic during the previous 12 weeks, as 4 to 6 cm long nematodes with long filamentous heads embedded in the mucosa of the caecum and large intestine. They are of negligible clinical significance. *Trichuris* spp. eggs are about 60 µm long, brown-coloured, thick-walled and extended oval-shaped, with a clear plug at both poles. Whipworm burdens may arise following both oral and percutaneous infection.
Anoplocephalid (tapeworm) eggs  100 µm	➧ *Monezia expansa* eggs are irregularly triangular shaped and about 60 µm across.
Coccidian oocysts  100 µm	➧ Most *Eimeria* spp. oocysts about 15 to 25 µm diameter (those of some species are slightly larger) and ovoid or spherical shaped, with refractive shells surrounding a mass of protoplasm. The oocysts of some species have a small pore at one end. Speciation of these oocysts is a specialist procedure, requiring leaving the faeces to stand for a few days to enable sporulation and accurate measurement of the oocysts.

Table 2.1: Eggs seen using standard salt floatation methods, which require differentiation. (From Sheep Flock Health – A Planned approach. pp156-157 (Sargison, 2008)

2.1.1.3.2.1 Egg morphometrics

Trichostrongyle eggs belonging to *Teladorsagia*, *Trichostrongylus, Haemonchus* and *Cooperia* genera are thin walled, oval shaped and between 70 and 100 μm long. The eggs may contain a morula of blastomeres or a pre-hatch larva, depending on the age of the faecal sample and their stage of development. *Nematodirus* spp. eggs are larger than the other trichostrongyle eggs, being 150 to 200 μm long, with a small number of discrete, large, round blastomere cells. *N. battus* has a dark, parallel-sided shell, while *N. filicollis* and *N. spathiger,* which do not have the same critical hatching requirements as *N. battus* and are of lesser clinical significance, have clearer shells with curved sides. While there are subtle differences in shape and size of some of *Teladorsagia*, *Trichostrongylus, Haemonchus* and *Cooperia* eggs, for example those of *H. contortus* are smaller, while those of *T. colubriformis* are slightly flattened, giving the curvature of their walls an asymmetrical appearance. However, these differences cannot be consistently or reliably differentiated on gross microscopic examination.

When required for this study, *Teladorsagia*, *Trichostrongylus, Haemonchus* eggs were differentiated using morphometrics, involving measuring of the length and breadth of 100 eggs, by comparing images of the eggs with an image of a calibrated graticule viewed under the same magnification. These values were plotted against each other, and compared with the co-ordinates for eggs belonging to known genera (Christie and Jackson, 1982) (for example, Fig 2.1).

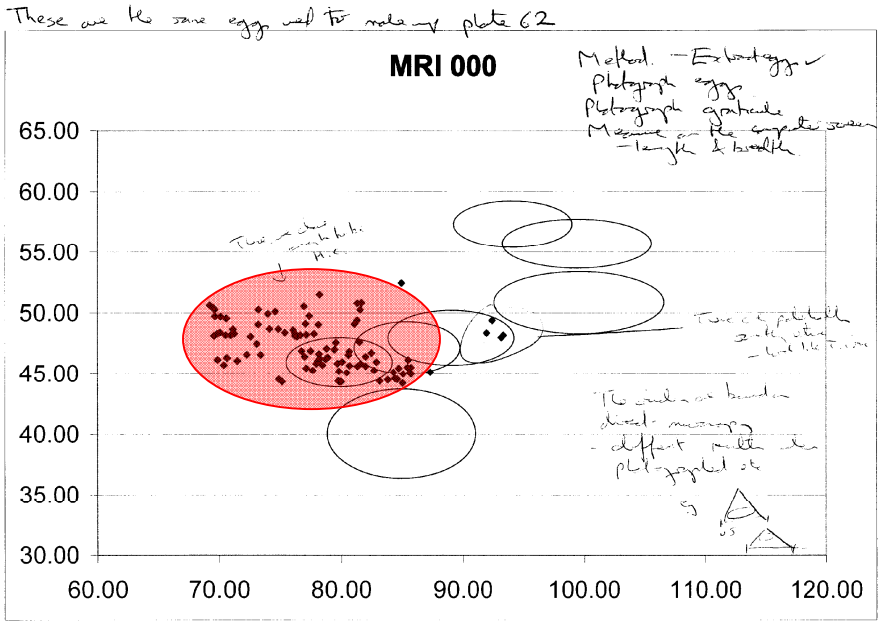


Fig 2.1: Plots of egg length (X axis) vs. egg breadth (Y axis) for individual eggs. Typical co-ordinates for *H. contortus* eggs are shown (red shading). (The oval shapes on the graph represent eggs of different species, but differ from the *H. contortus* area shown, because they were generated using direct microscopy, while an inverted microscope was used to generate the actual data.)

2.1.1.3.2.2 Fluorescent agglutinin staining

Trichostrongyle eggs of different genera can be stained with fluorescent agglutinins, which specifically bind to lectins on their surface. For example, *Haemonchus* spp. eggs can be identified using a fluorescein isothiocyanate-labeled peanut agglutinin (Palmer and McCombe, 1996).

During this study, trichostrongyle eggs were routinely stained with a fluorescein-labeled peanut agglutinin to support their identity as *H. contortus*. Eggs that had been extracted in saturated saline were washed by three cycles of suspension in tapwater, centrifugation at 203 g (1000 rpm) for 2 minutes, withdrawal of the supernatant and re-suspension of the egg-containing pellet in tapwater. Washed eggs were re-suspended in 1 ml of tapwater, in a 10 ml hard centrifuge tube. 10 µl of fluorescein-labeled peanut agglutinin (Vector Laboratories) were added, before leaving the suspension of eggs in water and

agglutinin to stand. After about 2 hours, the eggs were washed, using the same protocol, before examining under inverted fluorescent microscopy. The shells of >99% of *H. contortus* eggs showed intense green fluorescence using this procedure (Fig 2.2), while characteristic fluorescence was not observed in the shells of other UK trichostrongyle eggs.

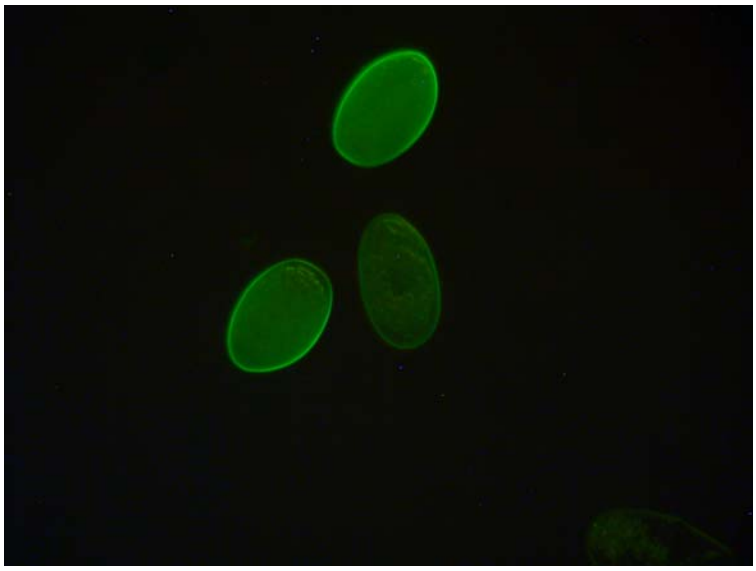


Fig 2.2: Fluorescein-labeled peanut agglutinin stained *H. contortus* eggs.

2.1.1.3.3 Coproculture and identification of third stage larvae

2.1.1.3.3.1 Bulk coproculture

Trichostronglye and strongyloid parasites can be identified to genus level by examining L_3 cultured from faeces. Fresh faeces were spread onto trays lined with a polythene bag and placed within another polythene bag perforated with air holes. Pelleted faeces were left intact and spread to a depth of about 3 cm, while pasty faeces were broken up and mixed with vermiculite. The faecal cultures were then incubated at 22^{o}C for about 10 days. L_3 were recovered by first flooding the faecal cultures for about two hours with tepid (~25^{o}C) water, before pouring through a coarse sieve while taking care not to break up the faecal mass. The filtrate was poured through a filter paper to trap any L_3 present. The filter paper was then submerged at the top of a jar full of tepid water, enabling the L_3 to move through it and to sediment to the bottom of the jar. After about

two hours, the Baermannised L_3 could be withdrawn from the base of the jar, and either examined immediately, or stored for several months in tissue culture flasks at 4°C or 8°C for *T. circumcincta* or *H. contortus* respectively.

2.1.1.3.3.2 Culture of L_3 on filter paper

An alternative method of L_3 culture was developed during the study to overcome problems of larval contamination of coprocultures (the method was broadly based on an unpublished Novartis Animal Health protocol). This method involved egg extraction from faeces, egg hatching (2.1.2.2), and inoculation of L_1 in about 100 µl of tapwater into a broth of about 5 x 10^8 colony forming units/ml of live *Escherichia coli* on a disc of cotton filter paper placed in a petri dish. The filter paper discs were cut from corrugated, fibrous, in-line milk filters (Hygia Cotton Discs for manual filtration Ø190 mm; Paul Hartmann AG D 89522 Heidenheim). Petri dishes were flooded with the broth containing the L_1 so that the peaks of the filter paper corrugations were just proud of the film of the *E. coli* broth. The petri dishes were then closed and placed inside a polystyrene box, containing a wet paper towel to maintain a moist environment and incubated at 24°C or 22°C for *Haemonchus* or *Teladorsagia* and *Trichostrongylus* spp. respectively. L_3 were recovered by Baermannising the filter paper.

The recovery rate of L_3 using this method was variable. Better results of up to 60% were achieved using a tox –ve O157 and OP50 *E. coli*, than using a different (unspecified) biotype. Better results were achieved when the *E. coli* were incubated for 24 hours at 37°C in LB broth, before inoculation of the filter paper. (The OP50 *E. coli* have a streptomycin resistant plasmid and were cultured in LB broth containing 12.5 µg/ml streptomycin). Most *H. contortus* larvae developed to the sheathed L_3 stage within 7 days, but recovery rates declined rapidly thereafter, presumably due to unfavourable conditions within the experimental setup. A similar method has been described, achieving better results using *E. coli* isolated from sheep faeces (Novartis Animal Health, standard operating procedure).

2.1.1.3.3.3 Identification of third stage larvae

Parasitic L_3 are generally enclosed in a protective sheath that extends to a point beyond their tail. Larvae that lack this sheath are either free-living, non-parasitic nematodes, which appear darkly stained with a double bulb-shaped oesophagus or *Strongyloides* spp. larvae, which are slender, with a simple oesophagus extending to about half of their length and a blunt tail that appears as if the tip has been broken off.

A drop of water containing larvae for identification was pipetted onto a microscope slide with a drop of helminthological iodine to kill and stain the sheathed L_3, and examined under a cover slip at 100 x magnification. A further sample of larvae was exsheathed by adding a drop of sodium hypochlorite solution and leaving for a few minutes before addition of the iodine and examination. The standard protocol involved the examination of about 100 sheathed and exsheathed L_3.

Parasitic nematodes with short sheathed tails were either *T. circumcincta* or *Trichostrongylus* spp.. These were differentiated by their morphology on the exsheathed sample. The exsheathed tails of *T. circumcincta* are smoothly rounded and asymmetrical, while those of *Trichostrongylus* spp. have one or two terminal tuberosities. Parasitic nematodes with medium sheathed tails were *Cooperia* spp., with square heads and two refractive bodies, or *H. contortus,* with bullet shaped heads. Long, filamentous sheath tailed nematodes were *Oesophagostomum* spp. or *Chabertia* spp. (Van Wyk and others, 2004).

2.1.2 The diagnosis of anthelmintic resistance

2.1.2.1 In vivo *diagnosis*

2.1.2.1.1 Faecal egg count reduction test (FECRT)

Most of the on-farm diagnoses of anthelmintic resistance that formed the basis for this study were based on the FECRT (Prichard and others 1980; Pomroy 1995). Pre-treatment FWECs were performed on between 10 and 12 sheep for each anthelmintic

group under investigation. The sheep were weighed and accurately drenched with the anthelmintic at its recommended dose rate. The sheep were marked to identify which anthelmintic group they were treated with and re-sampled between 7 and 14 days later. Post-treatment FWECs were performed. Reductions in FWEC were calculated from the arithmetic mean pre- and post-treatment counts. Reductions of less than 95 percent and 95% confidence intervals supported a diagnosis of anthelmintic resistance (Coles and others, 1992; Coles and others, 2006).

Anthelmintics were purchased from reputable sources, correctly stored according to the manufacturer's recommendation, and used within their expiry date. Dosing guns were checked beforehand by delivering aliquots of a preset volume into a calibrated container such as a syringe. Alternatively most of the sheep in this study were dosed using a syringe.

The timing of post-treatment sampling was partly determined by the anthelmintic under investigation. For imidazothiazole or tetrahydropyrimidine (levamisole or morantel) anthelmintics, samples were collected between 7 and 10 days post-treatment, because the efficacy of these drugs against EL_4 is poor (Grimshaw and others, 1996): trichostrongyle eggs seen in faeces after 10 days may have arisen from the maturation of female EL_4 which evaded exposure to a lethal dose of anthelmintic. For benzimidazole and avermectin anthelmintics, samples were collected between 12 and 14 days after treatment: FWECs can remain negative for up to 10 days after treatment with these anthelmintics, despite the presence of anthelmintic resistance, due to temporary suppression of egg laying by surviving resistant female nematodes (Martin and others, 1985); and trichostrongyle eggs may be identified in faeces from about 15 days after effective anthelmintic treatment associated with the minimum pre-patent periods of the trichostrongyle nematodes. Interpretation of FECRT results is potentially complicated for the persistent anthelmintic drug, moxidectin.

The grazing management of the animals between dosing and post-treatment sampling is unimportant. Most of the faecal samples were collected per rectum. Provided that sufficient animals were sampled on the first day, it was not always essential that every animal was re-sampled post-treatment. However, the probability of obtaining a sample from each animal was increased if the sheep were gathered shortly before sampling, rather than stood in yards for a prolonged period. Mean pre-treatment counts of more than about 350 epg were usually required to identify the presence of a low frequency of resistant nematodes.

The inclusion of a control group of untreated animals in addition to, or instead of performing pre-treatment FWECs is sometimes recommended (Coles and others, 1992). While this practice can improve the sensitivity of the FECRT under situations where FWECs are declining rapidly, for example during the autumn associated with the onset of host immunity to nematode parasites (Sargison and others, 2007a), or the possible influence of the age of the adult nematode population, it is usually unnecessary and impractical.

The sensitivity of FECRTs used in this study was improved by performing separate coprocultures and larval identification on pooled pre- and post-treatment samples corresponding to each anthelmintic under test. The percentage of each nematode genus in the samples can be determined, enabling FWEC reductions for each genus to be calculated, thus defining the resistant populations more accurately (McKenna, 1996; McKenna, 1997).

2.1.2.1.2 Critical efficacy test (CET)

The 'gold standard' test to confirm anthelmintic resistance is to infect nematode-free sheep with a known number of L_3, treat with a test dose of anthelmintic, after 14 days to test efficacy against EL_4, or after about 18 days to test efficacy against adult nematodes. Untreated control sheep are also required. Post treatment efficacies are calculated by

comparison of the total nematode counts of treated and control sheep (Coles and others, 2006).

2.1.2.2 In vitro *diagnosis*

During this course of study, egg hatch assays (EHAs) were used for the *in vitro* characterization of benzimidazole resistance, while larval feeding inhibition assays (LFIAs) and larval migration inhibition assays (LMIAs) were used for the characterization of ivermectin resistance. These assays first required the bulk extraction of nematode eggs from faeces.

2.1.2.2.1 Nematode egg extraction

Up to about 100 g of faeces was placed into a plastic bag and weighed. About 5 ml of tapwater was added per gram of faeces and the contents of the bag were then thoroughly emulsified using a stomacher apparatus. The contents of the bag were then mixed with more water to give a liquid suspension, which was washed over a series of sieves in order of 1 mm, 212 μm, 63 μm and 38 μm, collecting the retentate on the 38 μm sieve. This retentate was then washed into a bowl and then into flexible polyacrylamide centrifuge tubes using tapwater. The retentate suspension was then centrifuged for about 2 minutes at 203 g (1000 rpm), before removing the supernatant in each tube using a vacuum line, to leave pellets in about 0.5 ml of water. The pellets were then re-suspended in saturated NaCl solution and dispersed by gentle inversion before centrifuging the saturated sodium chloride suspension at 203 g (1000 rpm) for 2 minutes. The flexible centrifuge tubes were then clamped below the meniscus using artery forceps and the top layer poured onto a small 38μm sieve and rinsed with a gentle stream of tapwater from a wash bottle into a hard centrifuge tube. The retentate was then re-suspended with tapwater before centrifuging at 203 g (1000 rpm) for about 2 minutes and removing the supernatant using a vacuum line. Nematode eggs contained within the pellet that was left in the centrifuge tube, which was made up to 10 ml using tapwater, re-suspended and mixed. A 100 μl subsample was withdrawn into a 7 ml cuvette, which was then filled with saturated sodium chloride, capped and counted. The

egg count obtained by reading the cuvette was multiplied by 100 (10 ml/100 µl) to provide an estimate of the number of eggs present in the original sample. The FWEC of the original sample was estimated by dividing this count by its weight.

2.1.2.2.2 Egg hatch assay (EHA)

Nematode eggs extracted from fresh faeces as above, collected per rectum were washed by suspending them in water in a hard centrifuge tube, centrifuging for 2 minutes at 203 g (1000 rpm), withdrawing the supernatant using a vacuum line and then repeating the procedure two more times. The eggs in 100 µl of the mass extraction tapwater suspension were counted and the suspension diluted or concentrated by centrifugation and withdrawal of supernatant fluid so that it contained about 100 eggs/100 µl.

A 1000 µg/ml thiabendazole stock solution was prepared by dissolving 0.05 g thiabendazole (Sigma) in 20 ml dimethyl sulfoxide (DMSO) before making up to 50 ml in a volumetric flask with tapwater. Serial DMSO dilutions of 100, 60, 40, 20, 15, 10 and 5 µg/ml were made. (Note: the drug concentration in the made up solutions would have fallen about 3 fold during the first few days, but would have remained stable for many months thereafter when stored in the dark (Dave Bartley, *personal communication*).)

The base of a 24-well plate was scratched to provide reference marks. Next, 10 µl of each test drug dilution, 1890 µl of distilled water and 100 µl of water containing about 100 eggs was added in order to the wells, giving a dilution factor of 1/200 for the thiabendazole, with drug dilutions of 0.5, 0.3, 0.2, 0.1, 0.075, 0.05 and 0.025 µg/ml. Each drug dilution was used in two wells and DMSO controls were included. Eggs were added last, to ensure that they did not become coated in a higher concentration of drug than the final test concentration. Each well was checked under a dissecting microscope, before incubating for 48 hours at 22^{o}C or 24^{o}C for *Haemonchus* or *Teladorsagia*, respectively.

After 2 days, the contents of each well were killed, stained and fixed with a drop of helminthological iodine. The numbers of first stage larvae and eggs in each well were then counted to obtain the percentage of eggs hatched to L_1 in different concentrations of thiabendazole. These values were used to plot dose response curves, from which egg development (ED) values were calculated by Probit analysis (Minitab, ver. 13). ED_{50}, ED_{90} and ED_{99} were used as universal estimates of the concentration of drug required to prevent 50, 90 and 99% of the eggs from hatching. Benzimidazole resistance is generally deemed to be present if more than 50 percent of the eggs hatch in 0.1 μg/ml thiabendazole (Hunt and Taylor, 1989)

2.1.2.2.3 Larval feeding inhibition assay (LFIA)

The LFIA measures parasites' ability to feed after exposure to different concentrations of ivermectin.

Nematode eggs were extracted from fresh faecal samples as above, washed and incubated in 25 ml of tapwater in 10 cm diameter petri dishes for about 24 hours at 22°C or 24°C for *Haemonchus* and *Teladorsagia*, respectively, to yield L_1. The L_1 were then cleaned by transferring the contents of the petri dish to mini Baermann apparatus, comprising of 25 μm nylon mesh held over the end of sections of hard plastic tubing, and placed within the wells of a 6-well plate. The Baermann setups were incubated at 22°C or 24°C for about 1 hour, before removing the cylinders and mesh and transferring the contents of the wells of the 6-well plate to hard centrifuge tubes, filled to the 10 ml mark. The contents were then mixed, larvae in 100 μl aliquots were counted and the contents titrated so that each 100 μl contained about 100 L_1.

A 1000 μg/ml stock solution of ivermectin was prepared by dissolving 0.005 g of ivermectin (Sigma) in 5 ml of DMSO. Serial 1/10 dilutions were then made in DMSO, although different dilutions were also used.

Next, 1.5 ml (Eppendorf) microcentrifuge tubes were labeled with the final concentration of the ivermectin test dilution and arranged on a tray in two replicated rows. Ten µl of ivermectin test solution, 1390 µl of tapwater and 100 µl of tapwater containing about 100 L_1 were then pipetted into each tube in turn. The racks of tubes were left to stand for about 2 hours in a microbial extraction cabinet, with the lids open, during which time vials of frozen fluorescein isothiocyanate (FITC) labeled lyophilised *E. coli* were thawed. Seven µl of FITC labeled *E. coli* were then pipetted into each tube, before they were sealed, mixed and incubated for 18 hours at 22°C or 24°C for *Haemonchus* and *Teladorsagia*, respectively.

After about 18 hours the microcentrifuge tubes were removed from the incubator and centrifuged for about 3 minutes at 6000 rpm (~6000 g). Each tube was then opened and about 1 ml of the supernatant removed using a pipette. Ten µl, including the pellet containing L_1, were then transferred from the bottom of the tube onto a microscope slide and examined under an inverted fluorescent microscope fitted with a blue filter. Fed L_1 with a fluorescent pharynx and gut and unfed L_1 were counted to determine the percentage fed at each ivermectin dilution and to produce dose response curves from which larval feeding inhibition (LFI) values were calculated by Probit analysis (Minitab, ver. 13). LFI_{50}, LFI_{90} and LFI_{99} were used as universal estimates of the concentration of drug at which 50%, 90% and 99% of the L_1 do not feed.

Fig 2.3: Fluorescence of the pharynx and intestine of L_1 that have fed in the LFIA.

2.1.2.2.3.1 Preparation of FITC labeled lyophilised *E. coli* for use in LFIAs

FITC labeled lyophilised *E. coli* used in this study had already been prepared and stored at -20°C in 500 µl aliquots. One hundred µl of concentrated *E. coli* (2250 µg *E. coli* /ml) had been incubated with 1 mg of fluorescein isothiocyanate (FITC) in a 2 ml microcentrifuge tube at 20°C for 2 hours. The *E. coli* had then been centrifuged for 2 minutes at 13,000 rpm (~10,000 g) before removing the supernatant using a vacuum line. One ml PBS had then been added and centrifugation repeated two more times.

2.1.2.2.4 Larval migration inhibition assay (LMIA)

L_3 were transferred into 10 ml of tapwater in a hard centrifuge tube. Next, 750 µl of sodium hypochlorite (Milton fluid) were added to exsheath the L_3, while monitoring the process by examining a few drops of tapwater containing L_3 under a microscope. After about 2 minutes, once exsheathment was complete, the L_3 were washed in tapwater by three rounds of centrifugation, removal of the supernatant and re-suspension. The contents of the centrifuge tube were vigourously shaken after each re-suspension to untangle knots of L_3. After the final centrifugation, the tubes were filled to 5 ml.

Larvae in 10µl were counted and the amounts roughly adjusted to ensure about 120 L_3 in 100 µl.

Test dilutions of 0, 156, 312, 625, 1250 and 2500 µg/ml ivermectin were prepared from the stock 1000 µg/ml in DMSO solution. Reference marks were scratched onto the base of the wells of two 12-well plates. Ten µl of the test drug dilution, 1890 µl of tapwater and 100 µl of tapwater containing about 120 L_3 were added to each well, to give final drug concentrations of 0, 0.78, 1.56, 3.13, 6.25 and 12.5 µg/ml ivermectin. The plates were then incubated for 2 hours at 22°C or 24°C for *Haemonchus* and *Teladorsagia*, respectively, before the contents were transferred into the migration chambers comprising of 20 µm nylon mesh held over the end of sections of hard plastic tubing and incubated for 12 hours at 37°C. The migration chambers were then removed, everted into fresh wells and washed with 2 ml of tapwater to recover the non-migrated L_3 from the apparatus. Migrators were counted in the original 12-well plates, while non-migrators were counted on the second sets of 12-well plates. The proportion of migrating larvae at different drug concentrations were used to construct dose response curves from which larval migration inhibition (LMI) values were calculated by Probit analysis (Minitab, ver. 13). LMI_{50}, LMI_{90} and LMI_{99} were used as estimates of the concentration of drug at which 50%, 90% and 99% of the L_3 do not migrate.

2.1.2.2.5 Comment

With the benefit of hindsight, the use of tapwater when preparing anthelmintic dilutions for use in the bioassays could have led to problems with repeatability. Such problems could have arisen due to undetermined effects of differences in pH and in concentrations of minerals and chelating agents present in the tapwater on different dates on the drug concentrations and availability. These concerns may have been overcome by the use of buffered de-ionised water for the preparation of the initial drug concentrations.

2.1.3 Laboratory strains of *H. contortus*

2.1.3.1 Strains used for experimental studies

2.1.3.1.1 MHco3 (ISE)

The MHco3 (Inbred SE/ISE) strain was used as a reference ivermectin susceptible strain. The ISE strain was inbred from a highly heterogeneous (Otsen and others, 2001), out-bred population that had been maintained at the Moredun Research Institute since the 1950s, having been thought to have originated in Kenya, via the Glasgow Veterinary School. The process of inbreeding had involved dissecting the eggs from an adult female SE *H. contortus*, culturing these eggs for 7 days in worm free faeces, and injecting recovered L_3 into the rumen of a recipient sheep. The recipient sheep had been killed one week after they had started shedding trichostrongyle eggs. A single benzimidazole susceptible adult female *H. contortus* had been selected on the basis of its β-tubulin isotype 1 restriction fragment length polymporhism (RFLP) genotype. The progeny of this female was used to infect another recipient lamb and the process repeated fifteen times (Roos and others, 2004), to yield an inbred, but still genetically heterogeneous benzimidazole susceptible isolate. The resulting ISE strain was characterised using an egg hatch assay and β-tubulin isotype 1 RFLP genotyping (Roos and others, 2004). A version of this isolate provided by Dr Fred Borgsteede (Central Veterinary Institute, Lelystad, the Netherlands) to the Moredun Research Institute via Dr John Gilleard (University of Glasgow) and a new designation of MHco3(ISE) was given (this was to accurately identify this version of the original ISE strain and distinguish it from other versions being cultured in other laboratories). The MHco3 strain that has subsequently been maintained at the Moredun Research Institute by passage through donor sheep has subsequently been characterised *in vivo* and *in vitro* as susceptible to macrocyclic lactone, imidazothiazole and benzimidazole anthelmintics (Dave Bartley, *Personal communication*). It is the strain being used to provide DNA template for the *H. contortus* genome project (http://www.sanger.ac.uk/Projects/H_contortus/).

2.1.3.1.2 MHco4 (WRS)

The White River (WRS) strain *H. contortus* originated from the White River region in the Lowveld of the Transvaal in South Africa, as a field population that was resistant to benzimidazole, ivermectin and salicylanalide anthelmintics (Van Wyk and Malan, 1988). The WRS *H. contortus* has subsequently been characterised using *in vitro* and *in vivo* methods, confirming its resistance to benzimidazole, ivermectin and salicylanalide anthelmintics (Van Wyk and others, 1989) and susceptibility to levamisole and moxidectin (Oosthuizen and Erasmus, 1993; Gill and others, 1995; Jeannin and others, 1990; Le Jambre and others, 1995). The MHco4 strain (WRS) of *H. contortus* is now maintained at the Moredun Research Institute and used as a reference ivermectin resistant strain.

2.1.3.1.3 MHco10 (CAVR)

The Chiswick avermectin resistant (CAVR) strain arose as an serendipitous, extraneous, ivermectin resistant contaminant of a laboratory passaged *T. colubriformis* strain in Armidale, New South Wales, Australia (Le Jambre, 1993). The CAVR (MHco10) strain has subsequently been characterised and is resistant to ivermectin, but susceptible to benzimidazoles, levamisole and salicylanalide anthelmintics (Le Jambre and others, 1995). Macrocyclic lactone resistance in the CAVR strain appears to be independent of resistance to other anthelmintic groups (Gill and others, 1995) and inherited as a dominant trait (Le Jambre, 1993). Adult males appear to be more sensitive to ivermectin than adult females, which may be due to the physiology and behaviour of the males, or may imply a sex-linked inheritance of resistance (Le Jambre and others, 1995). The CAVR strain is morphologically different from other *H. contortus* isolates in that adult females lack a vulval flap (smooth morph) (Le Jambre and others, 1995) and may differ in its biology, for example its prepatent period of 24 days (Le Jambre, 1993) is longer than that of other *H. contortus* isolates and the period of inhibition of egg laying after ivermectin treatment is more protracted than in other ivermectin resistant populations (Le Jambre and others, 1995).

The origin of ivermectin resistance in the CAVR strain is unclear, and may differ from other ivermectin resistant populations. However, it is genetically highly divergent from the ISE strain (Redman and others, 2008), making it ideal for the analysis of genetic crossing experiments. A sample of L_3 of the CAVR isolate was provided to the Moredun Research Institute via Dr J. Gilleard (University of Glasgow) and this version was designated MHco10(CAVR) and has subsequently been maintained at the Moredun Research Institute by passage through donor sheep.

2.1.3.2 Maintenance of H. contortus *strains*

Worm free donor lambs were born and reared indoors. Their dams were all treated with 200 µg/kg moxidectin before housing, and the donor lambs were treated sequentially with 5 mg/kg of fenbendazole and 7.5 mg/kg of levamisole about 7 days before experimental nematode infection.

Worm-free donor male sheep were infected orally with between 5,000 and 7,500 L_3, taken from stocks held in tissue culture flasks into about 10 ml of tapwater. FWECs of the donors were usually monitored from about day 16 post infection, and webbing harnesses were attached to the sheep on about day 18 post infection. The wool over the breech was clipped prior to fitting the harnesses. Polythene bags were held against the breech and perineal region of the donor sheep, within cloth bags attached to the harnesses. The polythene bags were changed daily, allowing for the collection of between 800 g and 1.6 kg of faeces per day. This method was only used for male donor sheep, due to the inhibition of egg hatching caused by urine contamination that occurs if bags are attached to female sheep (Helle and others, 1989). Faecal samples were cultured in about 25 cm x 40 cm seed trays, in a dedicated culture room.

2.1.3.3 Cryopreservation

Pools of L_3 were cryopreserved for the purpose of future continued selection and analysis. L_3 were exsheathed by adding 750 µl of sodium hypochlorite (Milton fluid) to 10 ml of tapwater containing the L_3. Exsheathed L_3 were then washed by 3 cycles of

centrifugation at 203 g (1000 rpm) for 2 minutes, withdrawal of the supernatant and resuspension in 0.85% saline (neither buffer, nor glycerol were included in the freezing solution). Aliquots of 5,000 or 10,000 L_3 were then re-suspended in 0.85% into 2 ml cryopreservation vials, which were then snap frozen and stored in liquid nitrogen.

2.1.4 Surgical transfer

Live infective parasitic nematode L_3 cannot be sexed, so genetic crossing experiments between male and female nematodes with different genotypes necessitate the use of late L_4 or adult stages. Free living stages of the parasitic nematode life cycle can be grown to L_3 *in vitro*, but a satisfactory and straightforward method for maintaining and mating parasitic stages *in vitro* has not been identified (Stringfellow, 1986). Genetic crossing experiments therefore require the infection of sheep with late L_4 or adult male and female nematodes, which cannot be achieved orally and requires surgical implantation into the abomasum (Le Jambre, 1977; Le Jambre and others, 1999b), or proximal small intestine (Gopal and others, 2001) in the case of abomasal and intestinal parasites, respectively.

2.1.4.1 Donor sheep

The surgical transfer method used in this study first required the infection of worm-free donor sheep with L_3. Donor sheep were killed using an approved captive bolt method either 14 or 23 days after infection, to enable the recovery of late L_4/immature adult, or mature adult *H. contortus*, respectively. The abomasa were removed immediately and opened into a bucket containing warm (about 40^{o}C) 0.85% NaCl, by cutting along their greater curvatures. Once opened, the abomasal contents were gently washed off with 0.85% NaCl into the bucket. The abomasal contents and washings were poured over a 1 mm sieve and the retentate was then transferred into fresh 0.85% NaCl and maintained at about 37^{o}C. *H. contortus* were then picked from the abomasal contents and washings into warmed petri dishes containing RPMI 1640 tissue culture medium (Gibco; Invitrogen), before sorting into males and females.

2.1.4.2 Recipient sheep

Genetic crosses were performed by transferring the nematodes to be mated directly into the abomasa of worm-free recipient sheep. Anaesthesia was induced in the recipient sheep either by intravenous thiopentone injection, or using a halothane and oxygen mask. The sheep were then intubated and anaesthesia maintained using halothane and oxygen. The sheep were placed in left lateral recumbency and a 20 cm x 20 cm area was clipped and surgically prepared over the lower right abdomen. A 10 cm vertical incision was made through the skin, underlying fascia, muscle and peritoneum, over the right flank, midway between the last rib and pelvis and about 10 cm above the midline. The abomasum was then located and partially exteriorised, to enable a 1 cm diameter sub-serosal purse-string suture to be placed. A stab incision was then made in the centre of the purse-string, through which male and female nematodes were introduced into the abomasum in about 5 ml of RPMI per aliquot, using a blunt ended, 5 mm diameter glass pipette. The purse-string suture was then closed and the surgical incision repaired. Sheep were routinely injected with 1 mg/kg meloxicam (Metacam 20 mg/ml solution for injection; Boehringer Ingelheim) and 7 mg/kg amoxicillin/1.75 mg/kg clavulanic acid (Synulox ready-to-use injection; Pfizer) and closely monitored on completion of the surgery. No adverse effects were noted during the course of this study.

FWECs were monitored daily following surgical transfer, and harnesses and bags were fitted once they became positive.

2.2 Molecular biology methods

2.2.1 Preparation of lysates

2.2.1.1 Preparation of lysates at the Moredun Research Institute

The same method of lysis preparation at the Moredun Research Institute was used for bulk and single worm lysates of eggs, L_1 and L_3, with the exception of an initial exsheathment step. Exsheathment of L_3 involved their suspension in 10 ml of tapwater

and the addition of 750 µl of concentrated sodium hypochlorite (Milton fluid). (With the benefit of hindsight, the use of tapwater might have led to the transfer of ions or chelating agents that may have interfered with the lysate preparations and ultimately the PCR from those lysates.) The process was monitored by examining subsamples under a microscope, and normally took about 5 minutes, after which the L_3 were centrifugally washed in tapwater. Individual worm lysates were then made by transferring eggs, L_1, or L_3 in tapwater to a petri dish, from which they were withdrawn using a pipette set to 2 µl, into the wells of a 96 well plate containing 25 µl aliquots of lysis buffer (50 mM KCl; 10 mM Tris [pH8.3]; 2.5 mM $MgCl_2$; 0.45% Nonidet P-40; 0.45% Tween-20; 0.01% gelatine) and 5% proteinase K (Bioline). Bulk lysates were prepared by adding pellets of L_3 to 100 µl of lysis buffer/proteinase K in PCR tubes. The plates or tubes were then sealed and incubated in a water bath overnight at 60°C to allow lysis to occur. The lysates were then heated for 15 minutes at 95°C on a thermocycler (ABI 2700) to prevent further proteinase K activity and stored at -20°C.

(Purified DNA can be extracted from single or bulk worm preparations using proprietary miniprep kits to allow for more efficient amplification of DNA. However, this step was considered to be unnecessary and too laborious for the present study which involved very large numbers of preparations.)

2.2.1.2 Preparation of lysates at Glasgow University Veterinary School

Eggs or L_3 in water were centrifuged at 4000 rpm (~1500 g) to remove liquid and then resuspended in 1.5 ml Eppendorfs in M9 buffer (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl and 1 ml 1M $MgSO_4$ made up to 1 l in distilled water then autoclave sterilised). The eggs or L_3 were then centrifuged again at 4000 rpm (~1500 g) and the supernatant liquid removed. A further 200 µl of M9 buffer and 50 µl of sodium hypochlorite was then added to each Eppendorf, which was then left to stand for 5 to 10 minutes, before washing in M9 buffer using two cycles of suspension and centrifugation. The eggs or L_3 were then re-suspended in lysis buffer before transferring an aliquot onto the surface of an agar plate. Individual eggs or L_3 were picked individually from the agar plate into the

wells of 96 well PCR plates, containing 20 µl of lysis buffer and 5% proteinase K, using a platinum wire that was flamed between each nematode. The plates containing lysis buffer and proteinase K were always held on ice. Once plates were filled and sealed, they were placed in a freezer at -80^{o}C for 30 minutes to disrupt the nematode cells, and then transferred to a thermal cycler machine where they were heated at 60^{o}C for 98 minutes to allow lysis to occur, followed by 94^{o}C for 15 minutes to prevent further proteinase K activity. Lysates were stored at -80^{o}C.

2.2.1.3 Storage of lysates

A general tenet was that crude DNA lysates stored at -20^{o}C could only be freeze/thawed about 3 times before degradation of the covalent bonds between phosphorus, oxygen and carbon atoms that make up the repetitive sugar backbone of the DNA molecules, rendered them useless. Lysates stored at -80^{o}C were considered to be more stable (Libby Redman, *personal communication*). The potential problem of acceleration of lysis degradation by freeze/thawing was overcome by preparing replicate 1:10 or 1:20 dilution plates before storage. Furthermore, the efficiency of DNA amplification was generally improved when using diluted templates.

2.2.2 Polymerase chain reaction (PCR)

A section attempting to explain the principles that underpin the polymerase chain reaction is included, because while the PCR is regarded by molecular biologists to be a basic tool, it was to me a fundamentally new concept, given my background as a farm animal veterinary practitioner. Familiarisation with the PCR was important in the study underpinning this thesis, and addressing issues causing PCR failures accounted for a disproportionate amount of time. This section is based primarily on information drawn from a range of textbooks (for example, Griffiths and others, 2005) and is not referenced throughout.

2.2.2.1 The principles of natural cellular DNA replication

The term 'genome' refers to an organism's basic complement of deoxyribonucleic acid (DNA), which makes up the chromosomes that are present in every cell. DNA is the fundamental genetic material that determines biological function and caries information from one generation to the next. *H. contortus* nematode parasites are diploid, with a copy of the genome distributed amongst each set of 6 chromosome pairs (autosomal chromosomes are paired and females have a pair of sex chromosomes [XX], while males have a single sex chromosome [XO]). The term 'gene' used in this thesis relates to a chromosomal region that caries the information required to produce a single specific polypeptide, that may combine with other polypeptides to form a functional protein. Each chromosome in the genome carries a different array of genes. A gene is synonymous with an 'open reading frame' of DNA between start and stop codons. In fact, the region containing the coding sequence may be considerably longer than the actual coding sequence due to the presence of insertions of non-coding sequence (introns).

DNA molecules contain four nitrogen containing bases: thymine (T), cytosine (C), guanine (G), and adenine (A); in addition to a sugar and phosphate core. Large purines (G and A) in one strand are always matched by smaller pyrimidines (T and C) in the other, to give rise to the double helix, with specific hydrogen bonding between the complementary bases, so that A is always opposite T and G opposite C. The two DNA strands run in opposite directions. Conventionally, the top strand has a 5' OH group at the left end and is written 5' to 3' and the bottom strand runs in the opposite direction. (The lower strand does not need to be described, because both strands are complementary and its structure can be deduced.) RNA is very similar to DNA, but is single stranded and its backbone contains ribose rather than 2'deoxyribose.

The normal process of DNA replication that occurs during cell division first involves disruption of hydrogen bonds between the bases and separation of the two strands of the double helix, referred to as 'denaturation'. The two exposed nucleotide chains then act

as templates for the addition of free deoxynucleotide triphosphates (dNTPs) starting from their 3' ends, which are then joined together by the enzyme, DNA polymerase to form a new strand. The two daughter DNA molecules are identical to each other and to the parent molecule because of the base complimentarity.

Genetic function first involves the production of a complementary, single stranded messenger ribonucleic acid (mRNA) copy of one strand of the gene, starting at a specific promoter sequence and referred to as 'transcription'. In fact, T in the mRNA is replaced by uracil (U), which like T, always pairs with A. Ribosomes bind to a specific site at the 5'end of the mRNA molecule and read along its nucleotide sequence in groups of three successive bases, referred to as 'codons' until they reach a stop codon, translating the mRNA into a polypeptide. Sixty-four different codons are possible because there are four different bases and three bases in each codon. Some of these codons code for one of 20 different amino acids, and some amino acids are coded for by more than one codon (for example, AUU, AUC and AUA code for isoleucine).

Amino acids are synthesized into polypeptides on the ribosomes by transfer RNA molecules, which are complementary to the mRNA codon being read by the ribosome at that point in the assembly. The polypeptide is then folded, possibly in association with other subunits, to form a multi-subunit protein, which in some cases is further modified by glycosylation or phosphorylation, before its proper activity is manifested. To focus solely on genes as regions coding for proteins or polypeptides, ignores regions of DNA that while not coding for proteins, may be important for cell viability. For example, DNA sequences coding for RNA molecules such as ribosomal and transfer RNA; or regions that are important in gene regulation because they act as binding sites for regulatory proteins.

2.2.2.2 The principles of the in vitro *PCR*

PCR uses multiple copies of a pair of synthetic oligonucleotide primers, between about 15 and 20 bases long, that have been designed to have sequences that complement

primer binding sites at the 3' ends of the target region of DNA on two strands. The strands are separated by heating, referred to as 'melting', allowing the two primers to anneal to their binding sites, thus flanking the targeted sequence. Denaturation occurs over a short, specific temperature range that is influenced by the base composition of the target region, the midpoint of which is referred to as the 'melting temperature' (T_m) and annealing occurs as the DNA strands are cooled. A heat tolerant *Taq* polymerase (derived from *Thermus aquaticus* bacteria, naturally found in association with deep sea thermal vents) then moves along each DNA strand starting from each primer, synthesising the first set of complementary strands in the reaction. This process of extension occurs at a given temperature. These first two strands are longer than the targeted sequence, because they do not have a stop signal. The two duplexes are then melted and denatured again, exposing four binding sites, enabling the two primers to re-anneal to their respective strands at the 3' end of the target region, as they are cooled and *Taq* polymerase to synthesize two complementary strands when they are re-warmed to the extension temperature. These strands are precisely the same length as the target sequence, because each new strand begins at the primer binding site at one end of the target sequence and proceeds until it runs out of template at the other end of the sequence. Each new strand, therefore, begins with one primer sequence and ends with the other. The process of melting, annealing and extension is then repeated, each time producing two double stranded DNA molecules identical with the target sequence, giving rise to an exponential amplification of the DNA sequence between the two primers. The PCR is ideally suited to amplify target DNA segments less than 2 kb (Griffiths and others, 2005).

The temperature conditions required for the PCR can be created using automated thermal cycler machines.

2.2.2.2.1 Single product PCRs

Freeze dried primers were first made up to 100 μM, by adding nuclease free water according to the manufacturer's instructions, and then diluted into 10 μM aliquots, that

were used in the PCR at 1/10 of the total volume. Single product PCR assays conducted at the Moredun Research Institute involved the mixing of primers (1 μM final concentration) (different primer concentrations were used for pyrosequencing PCR), 10x buffer (1/10 of the total volume), $MgCl_2$ (between 1.5 mM and 2.5 mM final concentration), the four dNTPs (0.4 μM final concentration), nuclease free water and *Taq* polymerase (0.6 units per reaction). These reagents were stored at -20^{o}C, and thawed on ice prior to use (*Taq* polymerase was used straight from the -20^{o}C freezer). *Taq* polymerase was added penultimately and water was added last, to aid even mixing. Fourteen μl of this reaction mix and 1μl of template was pipetted into the wells of 96-well PCR reaction plates or PCR tubes.

The concentration of $MgCl_2$ determines the specificity of primer binding. The optimal concentration for each set of primers was determined by titration. The optimum annealing temperature for each set of primers was first estimated from the mean of the manufacturer's quoted T_m values based on their nucleotide composition, then refined using a PCR gradient block (Thermo Hybaid, MBS 0.2G) to test a range of temperatures.

Single product PCR assays performed at the Moredun Research Institute during the first 18 months of the study used Platinum® *Taq* DNA polymerase (Invitrogen) and a PCR Nucleotide mix 10mM each dNTP (Roche). PCR assays performed at the Moredun Research Institute during the latter part of the study used a Nova Taq™ Hot Start Master Mix Kit, in which the buffer, dNTPs and *Taq* polymerase were pre-mixed. Primers were supplied by MWG Biotech and molecular grade, nuclease free water was obtained from Fisher Scientific. PCR assays at the Moredun Research Institute were performed on an Applied Biosciences GeneAmp PCR System 2700 thermal cycler. Different conditions were used for different reactions.

Single product microsatellite PCR assays performed at the Glasgow Veterinary School involved the mixing of primers (0.5 μM final concentration), 11.1 buffer (9/100 of the

total volume), 10% Tween (Sigma Aldrich) (1/5 of the total volume), nuclease free water and *Taq* polymerase (0.5 units per reaction). These reagents were stored at -20^{o}C, and thawed on ice prior to use. Water was added first, *Taq* polymerase was added last and the reaction mix was gently agitated using a vortexer. Nineteen µl of this reaction mix and 1µl of template were pipetted into the wells of 96-well PCR reaction plates. The optimal concentrations of the reagents and PCR conditions had been predetermined (Erica Packard, *unpublished data*). (The 11.1. buffer was prepared using: 334 µl of 2M Tris HCl (pH 8.8); 166 µl of 1M ammonium sulphate; 67 µl of 1M $MgCl_2$; 7.2 µl of 100% 2-mercaptoethanol; 6.8 µl of EDTA (pH 8.0); 150 µl of 100 mM dATP; 150 µl of 100 mM dCTP; 150 µl of 100 mM dGTP; 150 µl of 100 mM dTTP; and 170 µl of 10 mg/ml BSA.) Single product PCR assays performed at the Glasgow Veterinary School used AmpliTaq® DNA Polymerase (Applied Biosystems) and dNTP Set 25µmol (Rovalab) nucleotides.

PCR assays at the Glasgow University Veterinary School were performed either on an Applied Biosciences GeneAmp PCR System 9700 thermal cycler, or on an Applied Biosciences Veriti 96 Well Fast Thermal Cycler. The thermal cycler conditions used for the single microsatellite PCRs were: 94^{o}C for 2 minutes; followed by 40 melting/annealing/extension cycles of 94^{o}C for 15 seconds, 54^{o}C for 45 seconds, and 72^{o}C for 1 minute; a final extension stage of 72^{o}C for 15 minutes; and a 4^{o}C hold.

2.2.2.2.2 Multiplex PCR

Microsatellite PCRs using primers with different green (HEX), blue (FAM) or yellow (NED) fluorescent labels, or using blue and green labels, or the same yellow labels, where the ranges of product sizes differs substantially can be performed simultaneously using a multiplex reaction.

Multiplex microsatellite PCR assays performed at the Glasgow Veterinary School involved the mixing of Master Mix (1/2 of the total volume) and Q solution (1/5 of the total volume) (Qiagen® Multiplex PCR Kit). A primer mix, with a final reaction volume

concentration of 2 μM final concentration and nuclease free water were added. Nineteen μl of this reaction mix and 1μl of template were pipetted into the wells of 96 well PCR reaction plates. The optimal concentrations of the reagents and PCR conditions had been predetermined (Erica Packard, *unpublished data*). The thermal cycler conditions used were: 95^{o}C for 15 minutes; followed by 40 melting/annealing/extension cycles of 94^{o}C for 30 seconds, 54^{o}C for 90 seconds, and 72^{o}C for 30 seconds; a final extension stage of 72^{o}C for 15 minutes; and a 4^{o}C hold.

2.2.2.2.3 Prevention of contamination of the PCR assay

The major potential source of contamination of PCR assays during this study was considered to be amplicons spread as aerosols or contact arising from the handling of the products of previous PCRs within a common airspace. This risk was reduced the Glasgow Veterinary School by the layout of the laboratories, whereby preparation of lysates, opening of PCR products and the PCR assay were all performed in dedicated, separated parts of the building. Furthermore, PCR assays were all performed in an ultraviolet sterilization cabinet. While PCR products at the Moredun Research Institute were generally not opened in the same laboratory used for PCR assays (for example, gel electrophoresis, or culture of transformed complement cells was always performed in a separate laboratory), some airborne amplicon contamination was unavoidable due to the necessary positioning of a pyrosequencer preparation area in the same airspace as the PCR workstation. The risk was minimised by the use of a designated ultraviolet sterilisation cabinet workstation, which was further treated before and after use with DNAzap (Ambion) to denature any contaminant DNA. The workstation contained a dedicated set of equipment (pipettes, racks and mini centrifuge) that was never removed from the hood, or added to. Only autoclaved, sterile consumables were introduced to the workstation.

Other sources of contamination associated with poor laboratory technique were addressed by taking utmost care when handling or pipetting reagents, for example by pulse centrifugation before opening vials and by using filter pipette tips. Reagents were

divided into aliquots in order that in the event of contamination, only a small amount would need to be discarded. Careful attention to detail was used during the preparation of lysates. Dedicated, clean protective clothing and disposable gloves were worn when performing PCR assays. The potential for contamination was monitored by always including negative lysis buffer and water controls in the PCR assay.

2.2.3 Methods used in this study to identify PCR products

2.2.3.1 Gel electrophoresis

Gel electrophoresis is based on the principle that when a charged molecule is placed in an electric field it will migrate towards the electrode with the opposite charge. DNA is negatively charged, so will migrate towards the anode. In a porous gel, the rate at which the nucleic acid molecule moves is determined by its ability to penetrate the structure of the gel. All DNA carries the same charge, irrespective of length, so for linear fragments of DNA, the rate of movement reflects their size. Agarose gels are used in this manner to separate nucleic acids greater than a few hundred base pairs, while polyacrylamide gels are required for smaller fragments, down to about 10 base pairs.

2.2.3.1.1 Agarose gels

Most of the PCR products from this study were identified using 1% (Moredun Research Institute) or 1.2% (Glasgow University Veterinary School) agarose gels. Gel Red or ethidium bromide was used to bind and stain the DNA in the respective agarose gels.

1% agarose/Gel Red gels were prepared by dissolving 4 g of agarose in 133 ml of 3x in H_2O Gel Red Nucleic Acid Stain Red (Biotium Inc), 8 ml of 50x TAE and 259 ml of distilled water, by heating in a microwave oven. The melted gel was then left to cool to about 40^{o}C before it was poured into a Perspex former, with combs to produce wells and left to set.

1.2% agarose/ethidium bromide gels were prepared by dissolving 3 g of agarose in 250 ml of 1xTAE (50x TAE was prepared making 242g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5M EDTA (pH 8.0) up to 1 litre in distilled water), by heating in a microwave oven. The melted gel was then left to cool to about 40^{o}C, before 12 µl of 10 mg/µl ethidium bromide (Sigma) were added and mixed by gentle swirling. The gel was then poured into a Perspex former, with combs to produce wells and left to set.

Once set, agarose gels were placed in horizontal electrophoresis gel tanks (Thistle Scientific), which were flooded above the level of the top of the gel with 1x TAE. Combs were removed to produce wells, into which a mixture of 1.5 µl of 'Blue Juice' loading buffer ('Blue Juice' loading buffer consisted of 0.25% bromophenol blue and 40% sucrose and was prepared by adding 2.5 ml of 1% bromophenol blue and 4 g of sucrose to 10 ml of distilled water) and 5 µl of PCR product was pipetted. A DNA ladder, such as DNA Molecular Weight Marker X (Roche) used at the Moredun Research Institute, was included in each row of wells. Depending on the size of the gel and the size of the DNA products being investigated, electrophoresis was carried out for between 30 and 45 minutes at between 90 and 130 volts to ensure adequate separation of the fragments and markers.

Stained DNA in the agarose gels was viewed and photographed under ultraviolet exposure using an Alpha Innotech AlphaImager™ 2200 (Moredun Research Institute) or Alpha Innotech Fluor Chem 5500 (Glasgow University Veterinary School) camera and Alpha Innotech MultiImage™ Light Cabinet.

2.2.3.1.2 Polyacrylamide gels

Polyacrylamide gels used for single strand conformational polymorphism (SSCP) analysis were run vertically in specially designed tanks (Thistle Scientific). Formers for 2 mm thick polyacrylamide gels were prepared by clamping ethanol-cleaned 20 x 15 cm

and 20 x 19 cm glass plates onto a framework of sealing plastic strips placed along three edges to form the sides and base.

Sufficient polyacrylamide for two gels was prepared by mixing 40 ml of distilled water, 40 ml of 30% w/v acrylamide/bisacrylamide (Severn Biotech), 20 ml 5x TBE, 622 µl of 10% ammonium persulfate (APS) (prepared by dissolving 0.1 g of APS (Sigma) in 1 ml of distilled water) and 88 µl of N' tetramethylethylenediamine (TEMED) (Fluka). The APS and TEMED were added last, before promptly pouring the gels between the glass plates, placed with the opening about raised about 10^0 from the horizontal. 20 well combs were then placed along the top edge of the gel. A small aliquot was set aside to monitor polymerisation of the gel. Once the gels had set, the bottom plastic former strips were removed, the glass plates and gel were clamped into horizontal electrophoresis tanks with the smaller plate facing to the back, the upper and lower portions of the tanks were flooded with 1x TAE, and the combs were removed. Heat treated PCR product and SSCP loading buffer was pipetted into each well, working quickly to avoid re-binding of the DNA strands and electrophoresis was performed for between 18 and 24 hours at a constant voltage of 100V.

Following electrophoresis, polyacrylamide gels were released from the glass plates and soaked in 1x Gel Red (133 ml of 3x in H_2O Gel Red Nucleic Acid Stain and 267 ml of distilled water), while gently agitating on a rocker tray for about 1 hour. Nucleic acid stained gels were then scanned under low density ultraviolet exposure using an AlphaImager 2200 (AlphaInnotech). Images were enlarged for analysis and background noise removed to sharpen the resolution using Quantity One software (v 4.5 Bio Rad Laboratories).

5x TBE was prepared by dissolving 54 g Trisbase, 27.5 g boric acid and 20 ml 0.5M EDTA (pH 8.0) in 1 litre of distilled water.

2.2.3.2 Pyrosequencing

Pyrosequencing can be applied to a variety of molecular approaches including SNP analysis, species identification sequencing and inter population polymorphism analysis. This study used pyrosequencing for SNP analysis to analyse the frequency of the F200Y and F167Y isotype 1 β-tubulin mutation.

2.2.3.2.1 Pyrosequencing PCR

Short (385 bp) biotinylated PCR products flanking codons 167 and 200 of the *H. contortus* isotype 1 β-tubulin gene were first generated using an unmodified forward primer (5' – GAG GCA TTC ACT TGG AGG AG) and a 5' biotin-labeled reverse primer (5' – CAT AGG TTG GAT TTG TGA GTT). These primers had been designed around the predetermined sequence of the isotype 1 β-tubulin gene using associated pyrosequencing software (Biotage) (Alison Donnan, *personal communication* citing background information from Von Samson-Himmelstjerna and others, 2007). An excess of the unmodified primer ensured that most of the biotin-labeled primer was used, and did not interfere with the subsequent pyrosequencing reaction. Each PCR reaction contained forward primer (0.4 μM final concentration), biotin labeled reverse primer (0.134 μM final concentration), 10x buffer (1/10 of the total volume), $MgCl_2$ (1.5 mM final concentration), the four dNTPs (0.4 μM final concentration), nuclease free water and *Taq* polymerase (0.4 units per reaction). Forty-five μl of this reaction mix and 5μl of template were pipetted into the wells of 96 well PCR reaction plates or PCR tubes. The thermal cycler conditions used were: 94^{o}C for 5 minutes; followed by 45 melting/annealing/extension cycles of 94^{o}C for 30 seconds, 58^{o}C for 30 seconds, and 72^{o}C for 45 seconds; a final extension stage of 72^{o}C for 10 minutes; and a 4^{o}C hold. PCR products were visualized on a 1.2% agarose gel to ensure that reactions had worked efficiently and to check for contamination using negative controls (for example Fig 2.4).

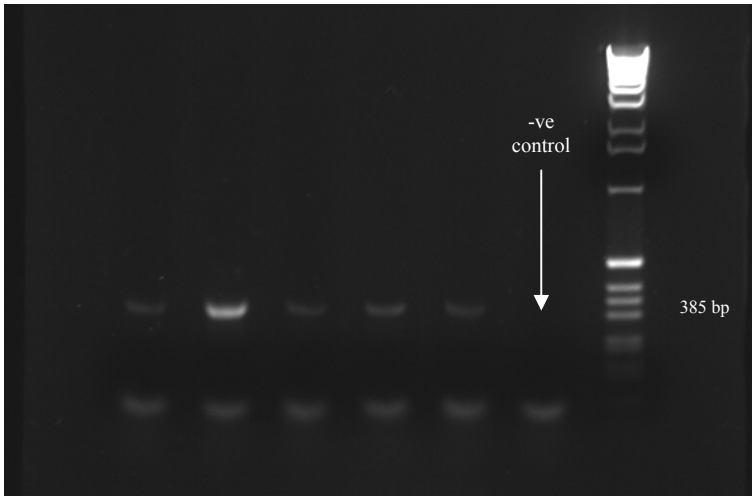


Fig 2.4: β tubulin (pyrosequencing) PCR products run on a 1% agarose gel. (The lower bands are probably unused primers.)

2.2.3.2.2 Pyrosequencing reaction

Next, the biotinylated PCR products were then bound to streptavidin-coated sepharose beads and denatured. Forty µl of PCR product were added to 3µl of streptavidin coated sepharose beads (Biotage) and 37 µl of binding buffer (Biotage) in the wells of 96-well PCR plates and agitated for at least 5 minutes on a plate shaker to allow binding of the biotin-labeled DNA to the beads. The bead-bound DNA was then processed using a dedicated sample preparation tool (Biotage) attached to a vacuum line, by washing in 70% ethanol, denaturation in 0.1M sodium hydroxide (Biotage denaturation solution) and washing to remove traces of sodium hydroxide (Biotage wash buffer). The beads were then dispensed into 96 well PSQ assay plates (Biotage) containing 40µl of 0.4 µM sequencing primer in annealing buffer (Biotage) per well. Nested sequencing primers were then hybridised to the DNA by heating the PSQ assay plates for 3 minutes on a thermal block (Biotage) placed on the stage of a thermal cycler pre-set to 80^{o}C, and then transferring them to the pyrosequencing machine and allowing their contents to cool to room temperature. The nested sequencing primers used to assay the F200Y and F167Y isotype 1 β-tubulin mutations were 5' – TAG AGA ACA CCG ATG AAA CAT and 5' – ATA GAA TTA TGG CTT CGT respectively.

The DNA samples were then analysed for the presence of relevant SNPs using a Pyromark ID (Biotage) pyrosequencer. The pyrosequencer software (Biotage) was informed of the expected sequence, including all possible mutations or indels. When the SNP run was started, an enzyme and substrate mix were dispensed into each reaction. This contained DNA polymerase, ATP sulfurylase, ATP luciferase, ATP apyrase and substrates. The substrates were adenosine 5' phosphosulphate (APS) and luciferin. Each nucleotide was dispensed into the well sequentially, and if it was not complimentary to the template sequence and incorporated into the new strand, it was degraded by the apyrase enzyme before the next base was added. The DNA polymerase catalysed the incorporation of each nucleotide into the complimentary strand by the same process as in regular PCR. This reaction released pyrophosphate (PPi) which was converted to ATP by ATP sulfurylase via the substrate APS. The ATP then drove the reaction of luciferin to oxyluciferin by luciferase, releasing light energy that was captured by a charge coupled device camera in the machine. The amount of light generated was proportional to the amount of PPi and ATP released, therefore the height of the peaks in the program output corresponded to the number of nucleotides of the same type incorporated in turn. The function of the apyrase enzyme was to degrade excess nucleotides and ATP continually, thus there was no interference with the next nucleotide incorporated. Where there were mutations to detect, the expectation of all possible complimentary bases was entered into the software. For example, in the case of the F200Y isotype 1 β-tubulin mutation, the third base expected was either a T or an A, therefore either an A or T nucleotide was predicted to be incorporated, but all four bases were still added one by one to the reaction. If a G or C nucleotide was incorporated, or the A/T signal was too weak, the sample would fail through the machine's in-built quality control. At the beginning of each assay, the pyrosequencer automatically released a non-complimentary nucleotide into the reaction to first ensure that there was no false positive signal. Other controls such as sequence primer only, water only, PCR lysis buffer negative controls and heterozygous positive controls were included on the assay plate (Stenhouse, 2007). The results were shown in a computer-generated printout. The sequence that appeared on the printout represented the actual sequence of

the target section of isotype 1 β-tubulin gene, because the reverse primers used in this study were biotinylated. If the forward primers had been biotinolated, then the printout would have been the reverse compliment of the gene sequence.

2.2.3.3 Ligation, transformation, cloning and sequencing

2.2.3.3.1 Isolation and purification of DNA

Gels containing PCR products of interest for cloning were viewed under ultraviolet light to enable relevant bands to be cleanly excised and transferred to 1.5 ml Eppendorf containers. DNA was then extracted from the gel using a proprietary QIAquik Gel Extraction kit (Qiagen) and following the manufacturer's protocol for a microcentrifuge method. Purified DNA was eluted into 30 µl of molecular grade water.

2.2.3.3.2 Ligation

PCR products have overhanging adenine bases at their 3' ends that can be stitched to complementary thymine bases at the 5' ends of a 50 ng/µl pGEM®-T (Promega) plasmid vector using a T4 ligase. Ten µl ligation reactions were set up using 5 µl of 2x ligase buffer, 1 µl of T4 ligase, 1 µl of PGEM®-T vector and 3 µl of insert DNA PCR product and incubated at 4°C for 48 hours.

2.2.3.3.3 Transformation

DNA contained within the plasmid vectors was incorporated into JM109 competent *E. coli* cells (Stratagene) to enable its cloning and expression. This process is referred to as 'transformation'. Competent cells were stored at -80°C and always handled carefully to avoid damage, by thawing slowly on ice and pipetting into pre-chilled, sterilised Eppendorf plastic ware. Forty µl of thawed competent *E. coli* cells and 3 µl of ligation reaction product were transferred directly to the bottom of labeled, chilled Eppendorfs, then left to sit on ice. (Any spare ligations were then stored at -20°C for future use.) After about 1 hour, the Eppendorfs containing the competent cells and ligations were heat shocked by plunging into a 42°C water bath for 1 minute to allow plasmids to enter the competent cells and be transformed. Next, 200 µl of thawed SOC medium, taken

under sterile conditions from a flamed bottle was pipetted into each Eppendorf of transformed plasmids, which were then gently shaken at 37^{o}C for about 2 hours to enable the competent cells to recover.

SOC is a very rich culture medium that was prepared by adding 1 ml of filter sterilised 20% glucose solution to 99 ml of a medium comprising of 20 g of tryptone, 5 g of yeast extract, 0.5 g of sodium chloride, 10 ml of 1M magnesium chloride and 10 ml of 1M magnesium sulphate made up to 1 litre with distilled water. The SOC medium was autoclave sterilized and stored at -20^{o}C.

2.2.3.3.4 Culture of transformed competent cells

The presence of a DNA insert in plasmids contained in transformed competent cells can be determined by a process of blue-white colour screening. The pGEM®-T vector plasmid contains a *lacZ* gene which is activated by IPTG to encode a β-galactosidase enzyme. The presence of an insert in the plasmid disrupts the *lacZ* gene and prevents the production of β-galactosidase. β-galactosidase cleaves X-Gal substrate to yield an insoluble blue product. Thus, a white to blue colour change is prevented in transformed bacterial colonies when a DNA insert is present in the plasmid.

Once the competent cells had recovered, a further 200 µl of SOC were added to each Eppendorf, before spreading 200 µl of the cell culture onto LB amp X-gal IPTG agar plates. The inoculated plates were then incubated overnight at 37^{o}C.

The following morning, culture plates were checked for a mix of blue and white bacterial colonies. The plates were then transferred to 4^{o}C for a few hours to increase the colour intensity of the blue colonies. White colonies, containing DNA inserts were then picked by stabbing sterile 1 ml pipette tips through the colony into the agar, and transferred aseptically to glass Universals containing LB 1x amp broth (20 µl of 500x ampicillin to 10 ml of LB broth). The cultures were then incubated overnight at 37^{o}C on a rocker apparatus.

Five hundred ml bottles of sterilised Luria Bertani (LB) agar were liquified slowly in a microwave oven and held at 55^{o}C in a water bath. Next, 1 ml of 500x ampicillin was added, to give LB 1x amp agar. (500x stock of ampicillin made up by dissolving an ampicillin 25 mg tablet (Stratagene) in 1 ml water and vigorous shaking in a vortexer.) The LB amp agar was then poured onto sterilized plastic petri dishes, the surface of the agar was flamed to remove air bubbles and the plates were left to set. Once set, the surfaces of LB amp agar plates were treated with 40 µl (40 mg/ml) of 5-bromo-4-chloro-3-indoly-beta-D-galactopyranoside (X-Gal) (Promega) and 40 µl of 1M isopropyl β-D-1-thiogalactopyranoside (IPTG) (Bioline). Prepared LB amp X-gal IPTG plates were then placed in a laminar flow cabinet for about 30 minutes before inoculation to remove condensation and enable evaporation of potentially toxic fumes.

2.2.3.3.5 Purification of DNA for sequencing

The following morning, Universals, containing the cultures were centrifuged at 3000 rpm (1000 g) and 20^{o}C for 10 minutes. Pellets containing double stranded plasmid DNA were then purified using a Wizard® Plus SV Minipreps DNA Purification System (Promega), following the manufacturer's instructions to clear the lysate, bind the plasmid DNA, wash and elute the plasmid DNA into 50µl of nuclease free water.

The concentration of double stranded DNA (ng/µl) in the final elution was estimated using a NanoDrop® ND100 spectrophotometer. Twenty µl were then submitted for sequencing, using SP6 and T7 universal primers designed for the pGEM®-T vector system. Any remaining DNA was frozen and stored at -20^{o}C.

2.2.4 Specific methods used in this study to identify molecular markers

2.2.4.1 PCRs to confirm nematode genus or species identity

Various molecular methods have been developed to confirm the species identity of most trichostrongyle nematodes. In this study the polycistronic regions of genomic DNA

coding for ribosomal RNA were routinely used in species specific PCRs to confirm the identity of *H. contortus*. Cistrons encoding rRNA occur in repeat units that are thousands of copies long, each separated by regions of non-transcribed DNA termed non-transcribed intergenic spacers (NTS). Each cistronic rRNA precursor transcript (5' - ETS - 18S - ITS1 - 5.8S - ITS2 - 28S - ETS - 3') contains external transcribed sequence (ETS) and precursor transcript for structural rRNA (18S, 5.8S and 28S), separated by non-functional internal transcribed spacers (ITS). Sequence comparison of these regions is useful because they are easy to amplify due to the high copy number of rRNA genes and because the ITS (Wimmer and others, 2004), and NTS (Dorris and others, 1999) regions in particular, show a high degree of variation even between closely related species.

The ITS2 primers that were used in this study (Forward: 5' – GTT ACA ATT TCA TAA CAT CAC GT; Reverse: 5' – TTT ACA GTT TGC AGA ACT TA) amplify product from *H. contortus* and *H. placei* but not from other trichostrongylid species (B. Wimmer, unpublished; John Gilleard, *personal communication*). The thermal cycler conditions used were: 94^{o}C for 2 minutes; followed by 40 melting/annealing/extension cycles of 92^{o}C for 30 seconds, 50^{o}C for 30 seconds, and 72^{o}C for 30 seconds; a final extension stage of 72^{o}C for 10 minutes; and a 4^{o}C hold. The expected PCR product size was 325 bp (for example, Fig 2.5).

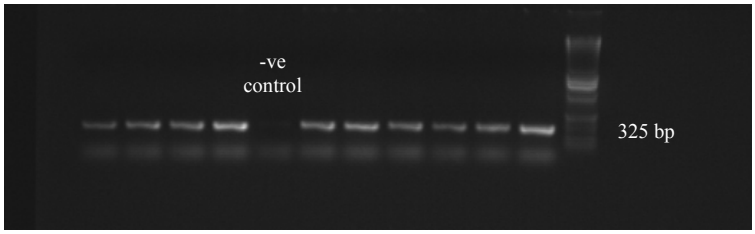


Fig 2.5: *H. contortus* ITS2 PCR products run on a 1% agarose gel. (Negative controls, for example using nuclease free water in the place of lysate template were routinely used to monitor or investigate contamination.)

NTS primers (Forward: 5' – GAG CTG GGT TTA GAC CGT CGT GAG; Reverse: 5' – AGC TCC AGT ATT TCC GCA GTT ATC C) were designed using a sequence

alignment of 18s and 28s rRNA genes from *C. elegans*, *Drosophila melanogaster*, *Saccaromyces pombe* and *Xenopus laevis* to select highly conserved regions of sequence (John Gilleard, *personal communication*). The thermal cycler conditions used were: 94°C for 3 minutes; followed by 40 melting/annealing/extension cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 150 seconds; a final extension stage of 72°C for 5 minutes; and a 4°C hold. The basis of species discrimination was in the size and or number of PCR products, the expected PCR product size for *H. contortus* being a double band of about 1.5 kb (for example, Fig 2.6).

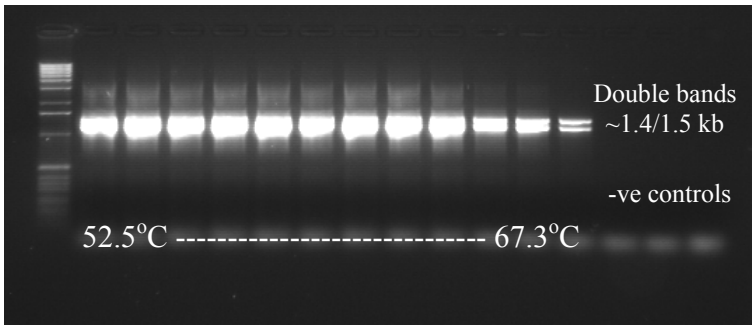


Fig 2.6: *H. contortus* NTS PCR products run on a 1% agarose gel using a gradient block (overexposed).

2.2.4.2 Microsatellites

Microsatellites are short repetitive sequences of DNA randomly dispersed throughout the genome. They comprise of tandem repeats of between one and six base pairs and constitute the most variable regions of DNA in the genome. The number of microsatellites in an organism is correlated with the size of the genome (Hancock, 1996). The high rate of mutation is a feature of repeated sequences, leading to polymorphism between individuals within a population. Microsatellites are found in both coding and non-coding regions of DNA, but the majority are found in non-coding regions (Hancock, 1995). The fact that microsatellites are polymorphic makes them suitable markers for studies of relatedness, such as human DNA fingerprinting. The fact that microsatellites are ubiquitous, means that they may be linked with genes affected by various selection pressures.

2.2.4.2.1 Microsatellite PCRs

The microsatellite markers used in this study had all been identified by bioinformatic screening of *H. contortus* expressed sequence tags (ESTs) and shotgun sequence databases (Table 2.2). Some of the primer sequences have been published (Redman and others, 2008), while some have only recently been screened (Libby Redman, *personal communication*) (Table 2.3). Forward primers were labeled with HEX (green), FAM (blue), or NED (yellow) fluorescent dyes (MWG Biotech). PCR products were visualized on a 1.2% agarose gel to ensure that reactions had worked efficiently, to check for contamination using negative controls and to estimate their DNA concentration, based on the strength of the relevant bands on the gel (for example, Fig 2.7).

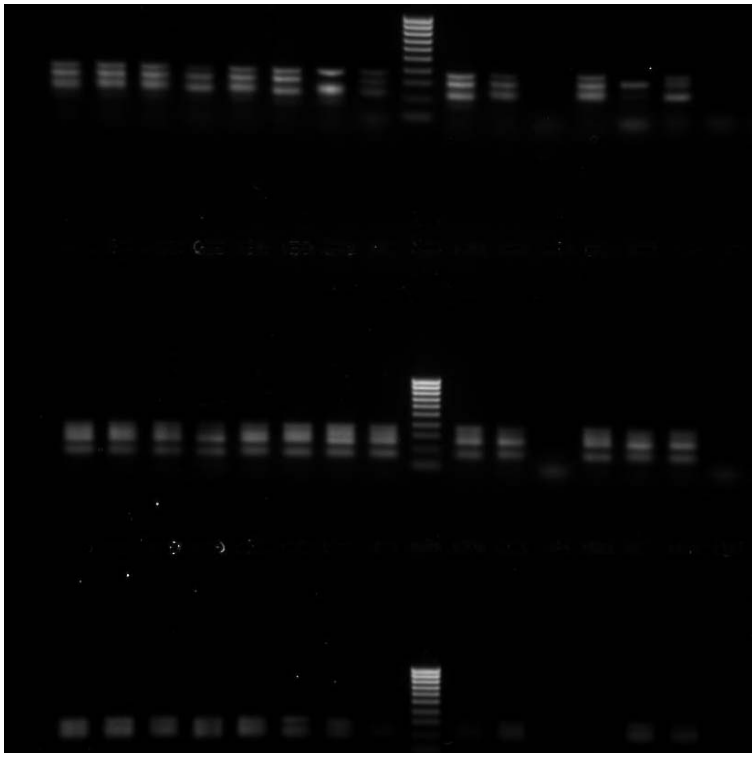


Fig 2.7: Multiplex microsatellite PCR products run on a 1.2% agarose gel using lysates of *H. contortus* L_3, to approximate the need to dilute product for Genescan.

Marker	Origin	Reference
8a20	Search of *H. contortus* shotgun sequence database (H_contortus.contigs.110305.gz) with Tandem Repeat Finder (Benson, 1999).	Redman and others, 2008
22co3	Search of *H. contortus* shotgun sequence database (H_contortus.contigs.110305.gz) with Tandem Repeat Finder (Benson, 1999).	Redman and others, 2008
3561	Identified in a *H. contortus* contig (139kb) and shown to be on Chromosome I by comparative analysis with *C. elegans.*	Redman and others, *unpublished data*
18210	Identified in a *H. contortus* contig (139kb) and shown to be on Chromosome I by comparative analysis with *C. elegans.*	Redman and others, *unpublished data*
26981	Identified in a *H. contortus* contig (139kb) and shown to be on Chromosome I by comparative analysis with *C. elegans.*	Redman and others, *unpublished data*
40506	New marker found by searching *H. contortus* contig database (H_contortus.contigs.012706.gz) with Tandem Repeat Finder (Benson, 1999).	Redman and others, *unpublished data*
60435A	New marker found by searching *H. contortus* contig database (H_contortus.contigs.012706.gz) with Tandem Repeat Finder (Benson,1999).	Redman and others, *unpublished data*
HcmsX142 (142509)	Identified in a *H. contortus* contig (393kb) and shown to be on the X chromosome by comparative analysis with *C. elegans*.	Redman and others, *In Press*
HcmsX146 (146945)	Identified in a *H. contortus* contig (393kb) and shown to be on the X chromosome by comparative analysis with *C. elegans*.	Redman and others, *In Press*
HcmsX151 (151069)	Identified in a *H. contortus* contig (393kb) and shown to be on the X chromosome by comparative analysis with *C. elegans*.	Redman and others., *In Press*
181881	Identified in a *H. contortus* contig (393kb) and shown to be on the X chromosome by comparative analysis with *C. elegans*	Redman and others, *unpublished data*
HcmsX182 (182762)	Identified in a *H. contortus* contig (393kb) and shown to be on the X chromosome by comparative analysis with *C. elegans*.	Redman and others, *In Press*
HcmsX256 (240993)	Identified in a *H. contortus* contig (393kb) and shown to be on the X chromosome by comparative analysis with *C. elegans*.	Redman and others, *In Press*
HcmsX337 (322017)	Identified in a *H. contortus* contig (393kb) and shown to be on the X chromosome by comparative analysis with *C. elegans*	Redman and others, *In Press*
Hcms25	Previously published, consistently amplifiable, unambiguous and polymorphic.	Otsen and others, 2000b
Hcms27	Previously published, consistently amplifiable, unambiguous and polymorphic.	Otsen and others, 2000b
Hcms33	Previously published, consistently amplifiable, unambiguous and polymorphic.	Otsen and others, 2000b
Hcms36	Previously published, consistently amplifiable, unambiguous and polymorphic.	Otsen and others, 2000b
Hcms40*	Previously published, consistently amplifiable, unambiguous and polymorphic.	Otsen and others, 2000b
Hcms94	Previously published, consistently amplifiable, unambiguous and polymorphic.	Otsen and others, 2000b

* Originally designated Hcms120

Table 2.2: Microsatellite markers used for the study.

Marker	Primer sequences
8a20	For HEX - CAA ACT TGA CCC GAC CTC TC Rev AGG GCC TTG CAC AAA CAT T
22co3	For NED – GAG CTT CAT TGA GAG AAT GGA ATT Rev AGG TCC TCA TAT ACG ATC AAC TAA
3561	For HEX – CCT ACA TGT CTC CCA TAT GTC Rev TTA GCG AAG TAA TAG CGT GCC
18210	For TET – TCA GGA GTT CGG CTT TTC AG Rev GTC AGC TGA GCT TCG TAA
26981	For FAM – ACA CTC TTA TCA CGC TAC CTG Rev CTC ACT TTC CCA GTC TTA TCG
40506	For FAM – CGT ATC CTA CTA GTA AGA TCC Rev ATG TGT GCC TTA TAC TTC TCG
60435A	For NED – CAT TTA CAC ACA TAC CCA TAT Rev GTA CGC ATC TAT AAG TGT TCT A
HcmsX142 (142509)	For HEX – ATT TCC AGG GCT ACG TAG TCC Rev AAG TTT CCA CTG AGT CAG TGC
HcmsX146 (146945)	For NED – CAA TTG TAC GAT GAT CGC CTG Rev CAA CTG TCA CAC ACG CAT AGC
HcmsX151 (151069)	For HEX – CAG ATT GTC CTC TAG TGG CTG Rev GTC ATC TTC TCT TCG TCG TCC
181881	For TET – TAC ATT CAG CCG GAA TGT GAC Rev TCC GGA GTC CTA CTT CAT CTG
HcmsX182 (182762)	For NED – GAC ACT TCA AGC TGT TCA GTC Rev
HcmsX256 (240993)	For NED – TCA CTC GTC ACA AAT CAC ACG Rev GTC GTT GTA ACT CGT TGA CC
HcmsX337 (322017)	For FAM – GTT GGC ATT TCC TGT CAT ACG Rev TTT AGT GTC AGC GCC TGT TTC
Hcms25	For ACA GGA GTT ATG AAT TTC CGG Rev FAM - GCT TCA GTT TGA ATT GCT TCC C
Hcms27	For ACA TAA ATC TAG GTA GGG TAG G Rev FAM – ACA GAA GAA CGA TCA GAA TCT C
Hcms33	For TET – ATA GCG GTT CGG AGG GGT Rev CCC CGT CAA ATA AAA GGC TAG
Hcms36	For HEX – GCA TAG CGG CAA GGA CGT ATG Rev CAT GAC GTA CTC TGG TTG TTC
Hcms40 (120)	For FAM – TCG ATA GTT GTC ACT TCC AA Rev TCG AAT CCT GAG TCT ACC GT

Table 2.3: Primer sequences for microsatellite markers used in this study.

2.2.4.2.2 Determination of microsatellite length

A subjective decision was made about whether or not to dilute PCR products, based on the strength of relevant bands on images of the electrophoretic agarose gel. Where strong bands were seen, products were diluted 1:50, 1:100, or 1:200 with nuclease free

water. Next, 1 µl of diluted or undiluted PCR product was added to a mixture of 19 µl of HiDi formamide (Applied Biosystems) and 0.2 µl of Genescan ROX 400 (Applied Biosystems) internal size standard marker in the wells of a 96 well PCR plate. The plate was then sealed with a flexible septum. DNA samples were denatured by heating for 5 minutes at 94^{o}C using a thermal cycler machine, and then stored at 4^{o}C until ready for analysis.

Microsatellite product sizes were determined using an Applied Biosystems/Hitachi ABI Prism 3130/Genetic Analyzer capillary sequencer in conjunction with Genescan software (Applied Biosystems). The output was in the form of a chromatogram for each sample, that was analysed using Applied Biosystems GeneMapper 4.0 software. The product size was noted for each sample and in cases where the peak height was low, or the shape of the peak was unclear, the process of sample preparation and capillary sequencing was repeated to ensure that the correct alleles were assigned.

2.2.4.2.3 Assigning of alleles based on microsatellite length

Microsatellite PCR product sizes were assembled into their corresponding previously determined binning ranges (agreed integer values, Libby Redman and John Gilleard, *Personal communication*), which were essentially their respective integers for analysis. These product sizes represented alleles, enabling data to be assembled to determine genotypes of individual or bulk nematodes. Alleles were entered into Microsoft Excel spreadsheets for analysis, for example using the GenAlEx version 6.1 add in software (Peakall and Smouse, 2006).

Individual genotypes were defined as homozygous null when samples could not be amplified for a specific microsatellite, despite numerous attempts (Grillo and others, 2006).

2.2.4.3 Single strand conformation polymorphism (SSCP)

SSCP involves the electrophoretic separation of single strands of nucleic acid on a non-denaturing polyacrylamide gel. Single stranded DNA is unstable and takes on a specific conformation that varies depending on the precise nucleotide sequence (for example, Blackhall and others, 2003). Hence, differences in the primary sequence, including subtle point mutations or deletions can affect the secondary structure and conformation of the single stranded DNA molecule. The conformation and length of the single stranded DNA fragment used determines the extent of the electrophoretic migration and appearance of each allele on a polyacrylamide gel. Under optimised conditions, the migration of these alleles and subsequent banding pattern is consistent and thus each allele can be assigned an arbitrary identity. This allows the frequency and combination of alleles to be compared between populations (for example, Blackhall and others, 1998; Ruiz and others, 2004). The method is more practical than the complexities associated with more traditional methods such as restriction fragment length polymorphism RFLP. Furthermore, unlike RFLP, which can only be performed on pooled material, SSCP can be used on individuals. SSCP works best on small lengths of DNA, between 100 and 500 bp.

2.2.4.3.1 SSCP PCR

SSCP was used in this study to identify mutations in individual nematodes that could be used as neutral markers to compare different populations of *H. contortus*. Primers were drawn from published literature for PCRs of polymorphic sections of GluCl α subunit (Forward: 5' – CCG ATT ATC CGC TTG ATG; Reverse: 5' – CCG TAT TGG TAA CTG ACG) (Blackhall and others, 1998b), GluCl β subunit (Forward: 5' – TTA TCA AGA CAG CCA ACG; Reverse: 5' – GGT AGT CGG GTT TTG TGA) (Blackhall and others, 1998b) and GABA subunit HG1 (Forward: 5' – GGT GAT GTC ATG GGT GTC; Reverse: 5' – TTG CTG CGA ATA CGA ATC) (Blackhall and others, 2003) genes. (These sets of SSCP primers were chosen because they had been reported as showing high levels of polymorphism in *H. contortus*.) The optimal thermal cycler conditions were: 95^{o}C for 4 minutes; followed by 40 melting/annealing/extension cycles

of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds; a final extension stage of 72°C for 5 minutes; and a 4°C hold. PCR products were visualized on a 1.2% agarose gel to ensure that reactions had worked efficiently and to check for contamination using negative controls (for example Fig 2.8).

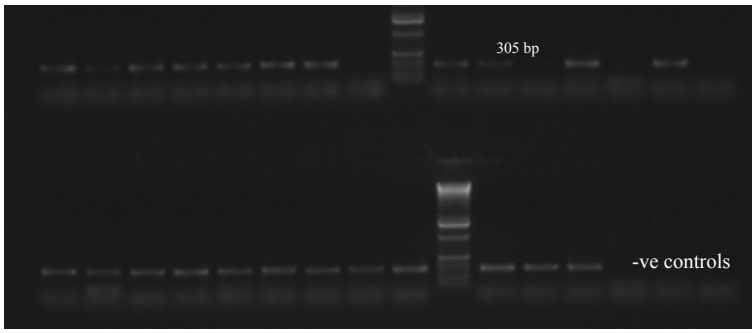


Fig 2.8: GABA Cl SSCP PCR products from lysates of individual adult female *H. contortus* heads run on a 1% agarose gel.

2.2.4.3.1 DNA strand separation

The DNA strands of the PCR products were separated by mixing in a formalin-based loading buffer and heating. Either 2 µl or 5 µl of PCR product (2 µl gave better resolution, possibly because of more room for the strands to fold in the relatively larger volume of buffer) and 5 µl of SSCP loading buffer were pipetted into PCR tubes, which were then heated to 100°C for 3 minutes by immersion in boiling water before plunging into ice to maintain strand separation. The denatured DNA was then loaded onto a 12 % vertical polyacrylamide gel and subjected to electrophoresis.

SSCP loading buffer consisted of 95% formamide, 10mM sodium hydroxide, 0.25% bromophenol blue (Sigma) and 0.25% xylene cyanol and was prepared from 47.5 ml of 95% formamide, 0.02 g of sodium hydroxide, 0.125 ml of 0.25% bromophenol blue and 0.125 ml of 0.25% xylene cyanol made up to 50 ml with distilled water.

Chapter 3: Development of methods to study the genetics of anthelmintic resistance

3.1 Introduction

The overall aim of the work presented in this thesis is to develop genetic approaches to study anthelmintic resistance and ultimately identify major loci carrying resistance-conferring mutations. The longer term aim of this study is to investigate alternatives to conventional candidate gene approaches to identify major molecular markers for ivermectin resistance in sheep parasitic nematodes. The prospect of a fully sequenced and annotated genome for the model parasitic nematode, *Haemonchus contortus*, provides a timely opportunity for the development of methods to enable genome wide comparisons between anthelmintic susceptible and resistant strains or isolates. The conventional candidate gene approach to identify mutations conferring anthelmintic resistance depends on pre-conceived ideas about its genetic basis, while comparison of the progeny of genetic crosses between anthelmintic susceptible and resistant isolates could enable a comparative search of the entire *H. contortus* genome for the major genes involved. Genetic crosses involving mass matings of populations of male and female parasitic nematodes are technically straightforward to perform, but meaningful genomic comparisons and genetic analysis is potentially confounded by the genetic diversity in the parent populations. This problem could be overcome if a genetic cross could be made between a single anthelmintic resistant and a single anthelmintic susceptible parent. Production of F_1 progeny of single male and female parent, free living, dioecious organisms is relatively straightforward, but sexual reproduction in parasitic nematodes occurs within their hosts, where it cannot easily be manipulated. Furthermore, comparison of the genotypes of anthelmintic susceptible and resistant progeny of a genetic cross requires the ability to reliably determine the resistance phenotype of individual parasites, which may not be straightforward.

It is possible that a single male and a single female *H. contortus,* when transferred directly into the abomasum of a worm-free recipient sheep, could survive for long enough to find each other, mate and shed eggs, and that only larvae hatched from these eggs could be recovered from faeces. Single *H. contortus* parent crosses have been referred to, but detailed genetic analysis to validate their success has not been published. The primary aim of this chapter was to determine whether or not such a successful genetic cross could be achieved and genetically validated following the surgical transfer of single male and female *H. contortus* into the abomasum of a recipient sheep. A critical objective of this work was to undertake detailed genetic analysis in order to validate any single nematode crosses that were performed. The detection of parasite eggs in the faeces of a recipient host in which a cross has been undertaken does not in itself prove success of a cross since a variety of artefacts and contamination events are possible (Fig 3.1).

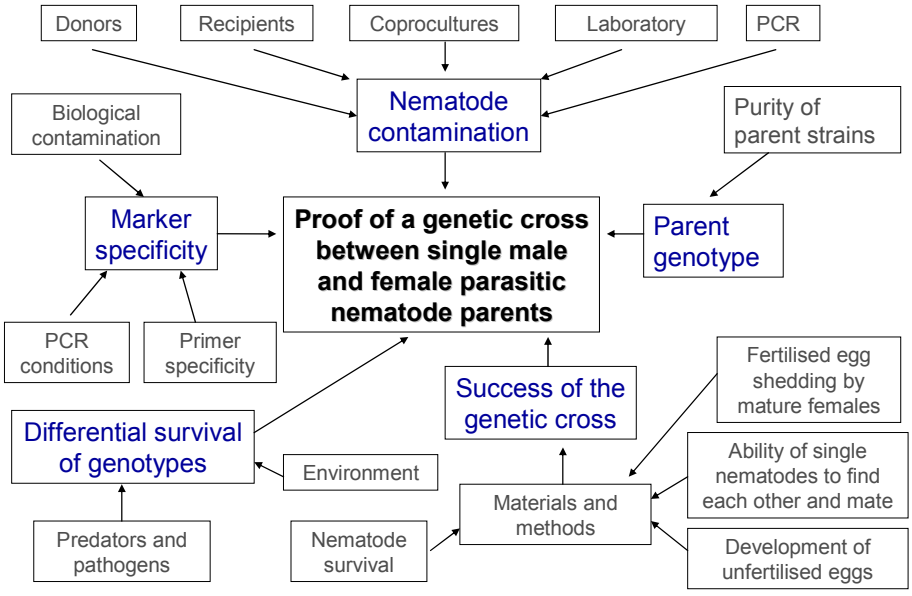


Fig 3.1: Issues that need to be addressed in order to provide proof of a successful genetic cross between single male and female *H. contortus* parents.

An understanding of basic reproductive biology and mating patterns is a prerequisite for the exploitation of genetic crosses to study anthelmintic resistance in parasitic nematodes. The mode of mating of most parasitic nematodes is either unknown, or assumed based on morphological or cytological evidence. It is now known that *H. contortus* is polyandrous and has a sex chromosome karyotype of two X chromosomes in females (XX) and a single X chromosome in males (XO) (Redman and others, *In Press*). The karyotype is 2n = 11 (♂) or 12 (♀). There is a generally held view that the parasite undergoes obligatory dioecious sexual reproduction, and that parthenogenesis or other forms of selfing such as hermaphroditism do not occur. This chapter aimed to investigate unknown aspects of *H. contortus* reproductive biology, such as the time between L_3 infection and the onset of sexual maturity, the duration of fertilised egg shedding by adult female nematodes following the removal of males, whether or not parthenogenetic development can occur in unfertilised ova, and how freely genetically divergent populations interbreed.

The analysis of a genetic cross between anthelmintic resistant and susceptible parents would depend on the use of Mendelian principles of inheritance where the different genotypic ratios can be predicted assuming independent segregation for unlinked loci. However, selection by environmental or biological factors may cause observed genotype proportions to vary from those predicted by simple Mendelian inheritance. An observational study was therefore performed to determine whether or not environmental factors influencing egg hatching, larval development and the storage of L_3 influenced the allele frequencies at a small number of neutral molecular marker loci.

Exploitation of single parent or mass mating crosses to study the genetics of anthelmintic resistance depends on the ability to accurately determine the resistance phenotype of both nematode populations and of individual nematodes. Phenotypically resistant nematodes can be reliably identified *in vivo* following anthelmintic treatment of their host, but susceptible phenotypes cannot easily be recovered in this way. Furthermore, some susceptible phenotypes may not survive anthelmintic treatment of the

host for reasons other than their resistance genotype. To-date, a diagnostic *in vitro* bioassay for ivermectin resistance has not been developed. The larval feeding inhibition assay (LFIA) is a useful experimental tool for the comparison and characterisation of different monospecific nematode populations, but may be of limited practical value to determine the *in vitro* resistance phenotype. Phenotypic expression of resistance may also be influenced by host parasite interactions, such as the effects of host age and/or immunity and drug bioavailability which can vary markedly between individuals. The chapter also aimed to investigate the practicalities involved with accurate determination of the ivermectin resistant phenotype and to determine the dominance of resistance in the bioassays used.

3.1.1 Chapter outline

The chapter addresses the issues presented in Fig 3.1. Firstly, preliminary studies are presented, describing the development of a single *H. contortus* parent genetic crossing method and its molecular genetic validation. A series of studies investigating previously poorly understood aspects of the reproductive biology of *H. contortus* is then presented. These studies describe: the duration of fertilised *H. contortus* egg shedding after removal of males; egg laying by day 14 female *H. contortus*; studies of interbreeding between different strains of *H. contortus*; and effects of environmental conditions affecting non-parasitic stages of *H. contortus* on molecular marker allele frequencies. Finally a series of experiments examining the phenotypic characterisation of reference isolates of *H. contortus* is presented. These studies describe: *in vitro* characterisation of the ivermectin resistance phenotype using the larval feeding inhibition assay; effects of donor age and immunity on the ivermectin resistance phenotype; and determination of the degree of dominance of the ivermectin resistance trait in the MHco3 (ISE) isolate. The materials and methods and results are presented independently. However, the studies are discussed together and summarised with reference to their importance in the development and analysis of genetic crossing approaches to identify genes associated with ivermectin resistance.

3.2 Materials and Methods

3.2.1 Development of a method to enable successful recovery of the progeny of single *H. contortus* parents

3.2.1.1 Surgical transfer

Male worm-free donor and recipient lambs were treated sequentially with 5 mg/kg of fenbendazole (Panacur 2.5%; Intervet) and 7.5 mg/kg of levamisole (Levacide 3%; Norbrook) 7 to 14 days before oral infection with L_3 or surgery. The worm-free donor lambs were infected *per os* on day 1 with either about 10,000 MHco3 or about 10,000 MHco4 *H. contortus* L_3. The donor lambs were euthanased on day 14 post infection and a single male MHco3 and a single female MHco4 late L_4/immature adult *H. contortus* were then surgically transferred into the abomasum of a male worm-free recipient lamb, within about 1 hour of recovery from the donor sheep. This protocol was followed twice, using three recipient lambs on each occasion (A, B and C on 14-2-06 and D, E and F on 18-9-06).

3.2.1.2 Animal parasitology

Harnesses and faecal collection bags were fitted to the recipient lambs from 3 days after surgery to enable daily faecal collection and coproculture. About 50 g of faeces were withdrawn from the collection bags and washed over a series of sieves to extract and count any eggs present. This procedure was followed daily for the first 14 days after surgery and 2 to 3 times weekly thereafter. The recipient sheep were maintained for up to two months, when they were euthanased, and any nematodes present in their abomasa were recovered and morphologically identified.

3.2.1.3 Infection of putative F_2 donor sheep

A worm-free donor lamb was infected with as many L_3 as practicable, recovered from the coprocultures from one of the recipient sheep from each round of surgical transfer

(recipients B and D), with the aim of producing F_2 progeny of the putative single parent genetic cross. A harness and faecal collection bag was fitted from 14 days after infection to enable daily faecal collection and coproculture and FWECs were monitored periodically. The donor sheep were maintained for about 2 months, before they were euthanased and any nematodes present in their abomasa and small intestines were recovered and morphologically identified.

3.2.1.4 Genus or species identification of putative progeny of the genetic crosses

Whenever practicable, supportive evidence of the identity of any eggs as those of *H. contortus* was sought by fluorescent agglutinin staining, or using egg morphometrics. Support of the *H. contortus* identity of L_3 recovered from coprocultures was sought by examination of larval morphology. DNA lysates were made of individual L_3 and PCRs performed using primers flanking the ITS2 and NTS loci to provide molecular support of their genus and species identity.

3.2.1.5 Molecular evidence for the validation of a single parent genetic cross

PCRs were performed on individual L_3 lysates, using primer pairs flanking a panel of 3 or 4 microsatellite markers (chosen from Hcms15, Hcms27, Hcms36, Hcms94, 8a20 and 22co3) and flanking the isotype 1 β tubulin codon F200Y SNP. Capillary sequencing in conjunction with Genescan software was then used to generate chromatograms for each microsatellite locus and the chromatograms were analysed using GeneMapper software. Allele frequencies interpreted from the Genescan traces were compared with reference data of allele frequencies from previously genotyped populations of MHco3, MHco4 and MHco10 *H. contortus* (Redman and others, 2008; Libby Redman, *data on file*). Isotype 1 β tubulin codon 200 alleles were determined by SNP analysis pyrosequencing. The *A*/*T* SNP at position 200 of the isotype 1 β tubulin gene resulting in a single amino acid substitution from tyrosine to phenyalanine (F200Y) was compared with data gathered throughout the study for MHco3 *H. contortus* and was used as an additional

well characterised marker. One test that a genetic cross between a single male MHco3 parent and a single female MHco4 parent was successful would be to determine that the F_1 progeny have genotypes at appropriate Mendelian ratios for a single pair mating. For example, the frequency of an allele (*a*) in the F_1 progeny of a mating between single nematode parents for a marker which has two alleles (*a* and *b*) should be 75% (for an *ab* x *aa cross*), 50% (for an *ab* x *ab cross*) or 25% (for an *ab* x *bb cross*). The frequency of heterozygotes (*ab*) in the F_1 progeny of a mating between single nematode parents for a marker with two alleles (*a* and *b*) should be 100% (for an *aa* x *bb cross*) or 50% (for *aa* x *ab* or *ab* x *bb* crosses). Two separate bulk DNA lysates were prepared of about 600 putative F_2 L_3. PCRs were performed in triplicate using primers flanking the Hcms15, Hcms25, Hcms27, Hcms22co3 and Hcms8a20 loci. The Genescan traces produced were compared with those for the reference *H. contortus* strains maintained at the Moredun Research Institute (MHco3, MHco4 and MHco10) and with field isolates.

3.2.1.6 In vitro *bioassays*

Egg hatch and larval feeding inhibition assays were performed using the methods described in chapter 2 on the putative F_2 progeny of one of the second round of single parent matings (recipient D).

3.2.1.7 Relevant information concerning an outbreak of haemonchosis in a sheep flock kept adjacent to experimental animals and facilities

An outbreak of haemonchosis was identified and investigated during June 2006, involving a flock of about 160 14 month-old Suffolk cross lambs kept at the Moredun Research Institute. The group of lambs had been introduced from two sources during the previous January and grazed on fields adjacent to the experimental animal accommodation and coproculture room. All of the lambs were dosed with 7.5 mg/kg of levamisole (Levacide 3%; Norbrook) during May, after which some were housed and the remainder were returned to the same fields. No signs of disease or ill thrift were reported prior to 5th June, when a weak and anaemic, ill thrifty (body condition score 1.0; range 1.0 – 5.0) lamb with a trichostrongyle FWEC of 8,800 epg was identified.

This lamb was euthanased and several thousand *H. contortus* were recovered from the abomasum on postmortem examination. Examination of a subgroup of 68 lambs showed that many, but not all were ill thrifty. Their mean FWEC was 759 (±SD 874) trichostrongyle epg. The whole group of 14 month-old lambs was treated sequentially with 7.5 mg/kg of levamisole (Levacide 3%; Norbrook) and 200 μg/kg ivermectin (Noramectin oral drench; Norbrook) on 12th June.

Day 14 post treatment FECRT efficacies of 100% were shown for levamisole, ivermectin and a combination of levamisole and ivermectin. LFIAs were performed on three occasions and an EHA was performed once. The mean LFI_{90} and LFI_{99} values (±SD) were 0.043 (±0.007) and 0.082 (±0.024) μg/ml ivermectin respectively and the ED_{50} value was 0.477 μg/ml thiabendazole, indicating a high level of benzimidazole resistance. In these respects the field isolate differed from the MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus* that were maintained in donor sheep and coprocultures at the Moredun Research Institute. PCRs were performed in triplicate on both of two bulk DNA lysates of 600 L_3 recovered from coprocultures, using primers flanking the Hcms15, Hcms25, Hcms27, Hcms33, Hcms22co3 and Hcms8a20 loci. The Genescan traces that were produced differed from the three experimental *H. contortus* strains, but were indistinguishable from a field isolate obtained during the investigation of a similar disease outbreak two years previously in a flock kept 20 miles away (Sargison and others 2007b).

The disease history and clinical findings suggest that this outbreak of haemonchosis arose following the maturation of overwintered hypobiotic EL_4, occurring soon after levamisole treatment of the group of lambs (the efficacy of levamisole against EL_4 is low, while the identification of ill thrift could have been related to the duration of the disease). Thus, at the time of the second round of single parent genetic crosses involving recipients D, E and F, there would have been very high levels of infective *H. contortus* L_3 contamination of pastures adjacent to the experimental animal accommodation and coproculture room at the Moredun Research Institute. This field

isolate of *H. contortus* was distinguishable from the MHco3 and MHco4 parent *H. contortus* strains used for the attempted single parent genetic crosses.

3.2.2 Reproductive biology and mating patterns of *H. contortus*

3.2.2.1 The duration of fertilised egg shedding after removal of males

An 11 month-old lamb that had been dosed 8 months previously with about 15,000 *T. circumcincta* L_3, but was no longer required as a donor because its FWEC had fallen below 50 epg, was dosed on day 1 with about 10,000 MHco3 (ISE) *H. contortus*. The donor lamb was euthanased on day 23 post infection and 200 adult female *H. contortus* were then recovered from its abomasum and surgically transferred into the abomasum of a male worm-free recipient lamb. A harness and faecal collection bag were fitted to the recipient lamb after surgery to enable daily faecal collection, egg extraction from about 50 g of faeces, and FWECs to be performed. Two aliquots each of about 100 eggs were transferred daily into tapwater in the wells of 24 well plates and incubated at 24^{o}C for 48 hours to monitor hatching. Support for the identity of the eggs as *H. contortus* was periodically sought by staining with fluorescent peanut agglutinin. This procedure was followed until day 41.

In an attempt to ascertain if female *H. contortus* would re-breed following a period of absence of males, the recipient lamb was dosed on day 41 (18 days after surgical transfer), with about 250 MHco10 (CAVR) L_3. Faeces were collected, FWECs performed and egg hatching monitored as before. The recipient lamb was euthanased on day 70, and *H. contortus* were recovered postmortem from the abomasum and counted. 24 female *H. contortus* with linguiform vulval flaps and 24 *H. contortus* lacking vulval flaps were transferred into 1 ml of RPMI tissue culture fluid in the wells of 24 well plates and incubated for 4 hours at 37^{o}C in 5% CO_2, before transferring to a 24^{o}C incubator for 48 hours to encourage egg shedding and monitor L_1 hatching. All of the eggs shed by individual female *H. contortus* and all of the L_1 that hatched were counted.

3.2.2.2 Egg laying by day 14 female H. contortus

In an experiment to ensure that day 14 late L_4 immature/ early adult female *H. contortus* would not shed fertilised eggs, a 5 month-old worm-free donor lamb was dosed in September 2006 (day 1) with about 10,000 MHco3 (ISE) *H. contortus* L_3. The donor lamb was euthanased on day 14 post infection and 200 day 14 late L_4 immature/ early adult female *H. contortus* were then surgically transferred into the abomasum of a male, 5 month-old worm-free recipient lamb (Recipient X). Two days after surgery, a harness and faecal collection bag were fitted to the recipient lamb to enable daily faecal collection, egg extraction from between 50 and 100 g of faeces, and FWECs to be performed. Coprocultures were periodically set up. Any eggs that were extracted were transferred into tapwater in the wells of 24 well plates and incubated at 24^{o}C for 48 hours to monitor hatching. Eggs were also periodically incubated at a range of temperatures between 22^{o}C and 37^{o}C. Support of the identity of the eggs as *H. contortus* was periodically sought by staining with fluorescent peanut agglutinin. Photographs were taken of the eggs and of a calibrated graticule using a standard light microscope to enable their accurate measurement. Recipient lamb X was euthanased on day 41, 17 days after surgical transfer, and all *H. contortus* were recovered from the abomasum. DNA lysates were prepared from adult female *H. contortus* heads and remaining *H. contortus* were stored in absolute ethanol for future study as required.

The procedure was repeated in June 2007, surgically transferring 155 day 14 late L_4 immature/ early adult female *H. contortus* into the abomasum of a male, 3 month-old worm-free recipient lamb (Recipient Y). Freshly extracted eggs and eggs that had been incubated at 24^{o}C for 24 or 48 hours were examined under differential interface contrast microscopy and photographed. Recipient lamb Y was euthanased on day 99, 75 days after surgical transfer and all *H. contortus* were recovered from the abomasum. 48 individual female *H. contortus* were transferred into 1 ml of RPMI tissue culture fluid in the wells of 24 well plates and incubated for 4 hours at 37^{o}C in 5% CO_2, before transferring to a 24^{o} incubator for 24 hours to encourage egg shedding. Eggs were

counted before the individual female nematodes and their egg broods were washed in PBS and cryopreserved.

DNA lysates were prepared of individual eggs and PCRs were performed using primer pairs flanking a panel of microsatellite markers (Hcms25, Hcms36, 3561, 40506 and 59736 for eggs from recipient X and Hcms25, Hcms27, Hcms36, Hcms120, Hcms22co3, Hcms8a20, X142, X146, X256, 18210 and 3561 for eggs from recipient Y). Capillary sequencing in conjunction with Genescan software was then used to generate chromatograms for each microsatellite locus and the chromatograms were analysed using GeneMapper software. The average number of alleles per locus and heterozygosity indices were calculated using Arelquin version 3.11 software (Nei, 1987; Excoffier and others, 2005). Data were defined as 'standard' rather than 'microsatellite' because the loci did not adhere to the stepwise mutation model (Redman, *In Press*).

3.2.2.3 Studies of interbreeding between different strains of H. contortus

Worm-free donor and recipient lambs were treated sequentially with 5 mg/kg of fenbendazole (Panacur 2.5%; Intervet) and 7.5 mg/kg of levamisole (Levacide 3%; Norbrook) 7 to 14 days before oral infection with L_3. Three primary 3 month-old donor lambs infected on the same day with either 5,000 MHco3 (ISE) *H. contortus* L_3, 5,000 MHco4 (WRS) *H. contortus* L_3, or 5,000 MHco10 (CAVR) *H. contortus* L_3. Harnesses and faecal collection bags were fitted from 20 days after infection to enable daily faecal collection and coproculture. FWECs were monitored daily to day 24 and weekly thereafter. DNA lysates were prepared from individual L_3 from the coprocultures of each primary *H. contortus* strain donor lamb.

Three 5 month-old donor lambs were then infected on the same day with mixtures of two different isolates; either 4,000 MHco3 (ISE) and 4,000 MHco4 (WRS) *H. contortus* L_3, 4,000 MHco3 (ISE) and 4,000 MHco10 (CAVR) *H. contortus* L_3, or 4,000 MHco4 (WRS) and 4,000 MHco10 (CAVR) *H. contortus* L_3 that had been cultured over the same period from the three primary donor lambs. FWECs were monitored daily from

day 17 to day 36. About 100 g of faeces was collected from each donor on days 21, 28 and 36. The faecal samples collected on days 21 and 36 were incubated for 8 days at 24^{o}C to culture L_3 and eggs were extracted directly from the faeces collected on day 28. Individual DNA lysates were prepared from 30 individual eggs, or L_3 hatched from eggs voided on days 21, 28 and 36. The three donor lambs were euthanased on day 37 and their total *H. contortus* burdens were estimated. 72 individual adult female *H. contortus* from each donor lamb were picked into 1 ml of RPMI in individual wells of 24 well plates and incubated for 24 hours at 37^{o}C and 5% CO_2. These female *H. contortus* and their egg and L_1 broods were then stored in absolute ethanol for future reference. Remaining adult *H. contortus* recovered from each donor were also stored in absolute ethanol.

PCRs were performed on the 30 DNA lysates prepared from individual eggs extracted from the faeces of each donor on day 28, and on 30 DNA lysates prepared from L_3 from the coprocultures of the three primary *H. contortus* strain donors, using primer pairs flanking a panel of microsatellite markers (Hcms8a20, Hcms33, Hcms25, Hcms27, Hcms40 and Hcms22co3). Capillary sequencing in conjunction with Genescan software was then used to generate chromatograms for each microsatellite locus and the chromatograms were analysed using GeneMapper software. Principal coordinate analysis was conducted using GenAlEx version 6.1 add in software (Peakall and Smouse, 2001) for Microsoft Excel to provide a schematic indication of the degree of interbreeding between the genetically diverse populations.

3.2.2.4 Effects of environmental conditions affecting non-parasitic stages of H. contortus *on molecular marker allele frequencies*

A donor lamb infected with MHco3 *H. contortus*, and with a FWEC of 3009 epg, was fitted with a harness and faecal collection bag. Faeces collected over a period of 24 hours were split into two equal aliquots of about 500 g. Coprocultures were set up for each aliquot at either 20^{o}C for 10 days or 37^{o}C for 5 days, following which L_3 were

harvested into tissue culture flasks. L_3 harvested from both the 20°C and the 37°C coprocultures were then stored at constant temperatures of 4°C, 22°C or 37°C.

Aliquots of L_3 from the tissue culture flasks were Baermannised and bulk DNA lysates were prepared of about 600 live L_3, following coproculture (day10), and at intervals thereafter (days 24, 58, 116 and 212). PCRs were performed on the bulk DNA lysates using primer pairs flanking a panel of microsatellite markers (Hcms25, Hcms36, 3561, 40506, 59737). Capillary sequencing in conjunction with Genescan software was then used to generate chromatograms for each microsatellite locus and the chromatograms were analysed using GeneMapper software.

3.2.3 Phenotypic characterisation of isolates and strains of *H. contortus*

3.2.3.1 The larval feeding inhibition assay

The LFIA was used to characterise the ivermectin resistance phenotype *in vitro* of the MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus* used throughout this study. The assay was performed as described in the general materials and methods chapter and the numbers of fed and unfed L_1 in each replicated concentration of ivermectin was used to produce a dose response curve. The discriminatory concentrations of ivermectin at which feeding was inhibited in 90 % and in 99% of L_1 (LFI_{90} and LFI_{99}) were calculated by Probit analysis using Minitab ver. 13 software. A preliminarily assessment of the accuracy and reliability of the LFIA to determine the ivermectin resistance phenotype *in vitro* was sought by comparison of the results of the assays performed using L_1 hatched from eggs voided by 4 x MHco3 (ISE), 2 x MHco4 (WRS) and 2 x MHco10 (CAVR) *H. contortus* donor lambs over a period of about one year.

Consistently low levels of L_1 feeding in the LFIA have previously been recorded, mostly occurring throughout the winter months and improving during the spring (Yvonne

Gordon, *Personal communication*). The problem occurs at all drug concentrations and is also seen in drug-free controls. Similar problems occurred throughout the course of this study. Low levels of L_1 feeding in drug free controls were usually accompanied by fungal contamination, leading to clumping of L_1, visible growth of fungal hyphae over the tegument of the L_1, internal fungal growth and lysis of the L_1, and/or morphological distortion of the L_1 (Fig 3.2).

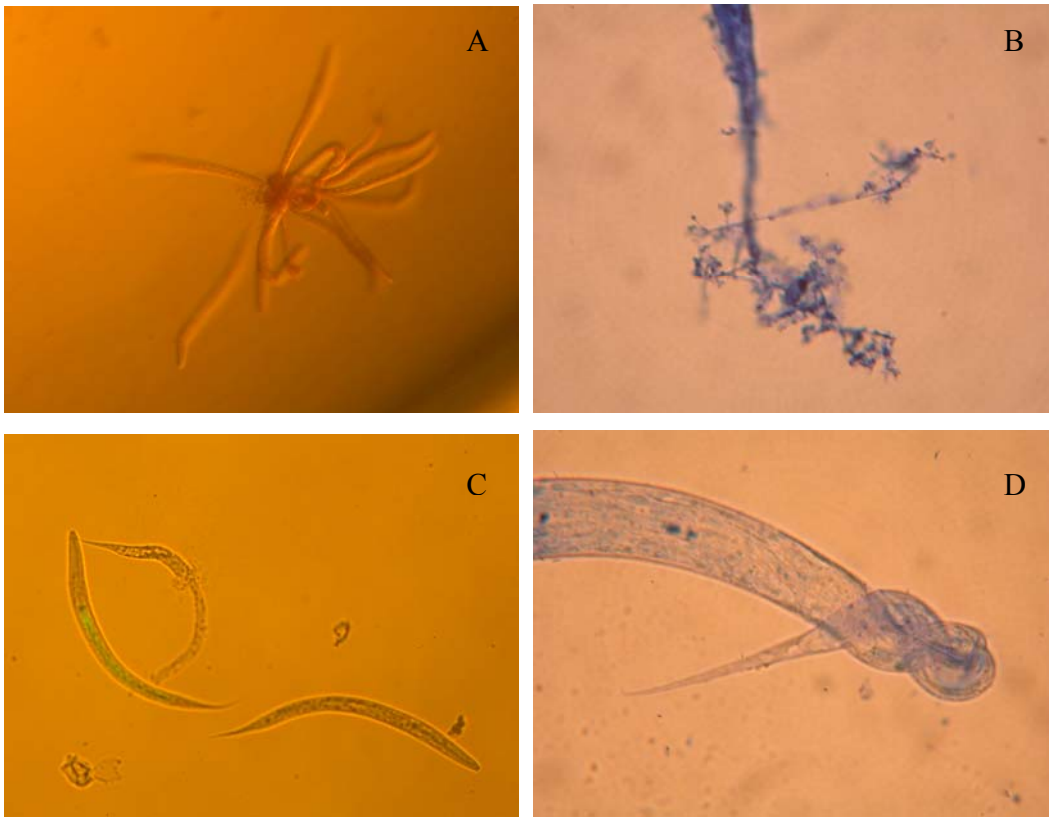


Fig 3.2: Effects of fungal contamination in the LFIA on L_1. **A:** L_1 clumped together, attached by their tails, associated with the presence of fungal hyphae. **B:** Fungal hyphae growing over the tail of a L_1. **C:** A normal, fed L_1, a partially lysed L_1 parasitised by fungal hyphae and an unfed L_1. **D:** Distorted tail of an L_1 probably caused by pathogen infection.

Larval feeding in the water control conditions could be influenced by the fitness of the L_1, or the availability of food (lyophilised *E. coli*). The fitness of the L_1 could be associated with: conditions influencing the parent nematodes or eggs within the sheep

host; environmental factors such as pH, temperature or oxygenation during egg extraction, egg hatching, or larval incubation; mycotic or bacterial contamination; or the presence of mycotoxins or other inhibitory factors. The availability of lyophilised *E. coli* could be influenced by: the quantity fed; the way in which it is presented; or its palatability, which could in turn be affected by environmental conditions. A series of observations was therefore made on the effects of environmental conditions on the LFIA, comparing between the MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* the effects of: storing faeces at 5^{o}C for 24 hours before egg extraction; water temperature during egg extraction; and the temperature, pH, oxygenation (performing the assays on 6 or 24 well plates, or with positive aeration) and source (distilled or tapwater) of the water used for egg hatching and larval feeding.

3.2.3.2 The effects of donor age and immunity

Throughout the course of this study, problems arose associated with the use of older donor or recipient sheep. For example, larval feeding in the LFIA was poor during the winter months when older donor sheep were used, and improved in the spring when young lambs became available. The success of surgical transfers was generally poorer when older donor and recipient sheep were used, and optimal when young lambs were used during spring and early summer months. These problems were not necessarily solely due to the age of the donor sheep, and may have been confounded by extrinsic seasonal factors. An experiment was, therefore, designed to compare aspects of *H. contortus* parasitology in older 14 month-old worm-free donor sheep with young 3 month-old lambs.

Twelve 14 month-old Greyface ram lambs (hoggs) and three 12 week-old crossbred ram lambs, that had been born indoors and housed for their entire lives to ensure a worm-free status were treated sequentially with 5 mg/kg of fenbendazole (Panacur 2.5%; Intervet) and 7.5 mg/kg of levamisole (Levacide 3%; Norbrook), 7 to 14 days before oral infection with L_3. The hoggs were all in the same good body condition (score 4: range 1-5) and had similar bodyweights (60 – 65kg) and the lambs were evenly well grown.

The sheep were randomly allocated to three groups of 4 hoggs (A - D) and one lamb (E). The three groups of sheep were orally dosed on day 1 with 5,000 MHco3 L_3, 5,000 MHco4 L_3, or 5,000 MHco10 L_3. Suppression of the host immune response to *H. contortus* infective L_3 and adult nematodes was attempted using weekly injections of methyl progesterone acetate (MPA). Two hoggs from each group (A and B), were treated with 80 mg (~ 1.3 mg/kg) of MPA i.m. (2ml of Depo-Medrone V; Pfizer), three days before oral infection with L_3 and at irregular intervals thereafter on days 5, 12, 19, 26 and 33.

The FWECs of each hogg or lamb were monitored daily throughout the course of the experiment between days 17 and 45. Individual EHAs were performed on days 20, 25, 31 and 40 using eggs voided in the faeces of each hogg or lamb. Individual LFIAs were performed using L_1 hatched from eggs voided in the faeces of each lamb on days 24, 27, 31, 34 and 40. On days 21, 28 and 41, subsamples of about 10 g of faeces were weighed into plastic trays, which were then placed on larger trays inside perforated polythene bags and incubated at 24^{o}C for 10 days to culture L_3. L_3 were recovered from the coprocultures by Baermannisation and then counted, to provide an estimate of the success of development of eggs voided by each hogg or lamb to L_3. Harnesses and faecal collection bags were fitted to 5 sheep on day 44 to determine the weight of faeces produced over a 24 hour period and enable the estimation of daily faecal worm egg outputs. The 12 hoggs and 3 lambs were euthanased on day 45, following which total postmortem abomasal worm counts were performed.

3.2.3.3 Determination of the degree of dominance of the resistance trait in the MHco4 (WRS) isolate

Concurrently with the first attempt to achieve a single *H. contortus* parent genetic cross, 20 male MHco3 (ISE) and 20 female MHco4 (WRS) day 14 *H. contortus* late L_4/immature adult *H. contortus* were surgically transferred into the abomasum of a male worm-free recipient lamb. A harness and faecal collection bag was fitted to the recipient

lamb from 3 days after surgery (day17) to enable daily faecal collection and coproculture of F_1 L_3.

Two worm-free donor lambs (A and B) were infected with about 5,000 F_1 L_3 recovered from the coprocultures of the recipient lamb. Harnesses and faecal collection bags were fitted to the F_2 donor lambs from 18 days after L_3 infection (day 18) to enable daily faecal collection and coproculture of F_2 L_3. The F_2 donor lambs A and B were treated with 200 μg/kg and 100 μg/kg ivermectin respectively on day 37.

The recipient lamb was euthanased on day 198 (184 days after surgical transfer) and the two F_2 donor lambs were euthanased on day 113 after L_3 infection. Adult *H. contortus* were recovered postmortem from the abomasa and counted. DNA lysates were prepared from the heads of the parental adult *H. contortus* recovered from the recipient lamb, and adult F_1 *H. contortus* recovered from the two F_2 donor lambs were both cryporeserved and stored in absolute ethanol.

FWECs of the recipient and F_2 donor lambs were monitored throughout the course of their *H. contortus* infections. LFIAs were performed using L_1 hatched from eggs extracted from the faeces of the recipient lamb on days 49 and 78 and using L_1 hatched from eggs extracted from the faeces of the F_2 donor lambs on days 34, 36, 48, 60 and 105. EHAs were performed using eggs extracted from the faeces of the recipient lamb on day 55 and using eggs extracted from the faeces of the F_2 donor lambs on days 36, 57 and 59. Whenever possible, LFIAs and EHAs were performed concurrently using contemporary MHco3 (ISE) and/or MHco4 (WRS) *H. contortus* L_1 or eggs.

Support of a successful genetic cross between 20 MHco3 (ISE) and 20 MHco4 (WRS) *H. contortus* parents was sought using microsatellite DNA fingerprinting. PCRs were performed on 37 individual L_3 lysates, using primer pairs flanking a panel of 4 microsatellite markers (Hcms15, Hcms27, Hcms94 and Hcms22c03). Capillary sequencing in conjunction with Genescan software was then used to generate

chromatograms for each microsatellite locus and the chromatograms were analysed using GeneMapper software. Allele frequencies interpreted from the Genescan traces were compared with reference data based on individual lysates of MHco3 (ISE) , MHco4 (WRS) and MHco10 (CAVR) *H. contortus* (Libby Redman, *data on file*).

Determination of the degree of dominance or recessivity of the anthelmintic resistance trait was based on the Mendelian principle that if the parental genotypes are monomorphic and homozygous (RR and SS) at a single locus or loci conferring anthelmintic resistance, then the F_1 population will be heterozygous (RS) and phenotypic expression of resistance in the F_1 population indicates that the R allele is dominant. Phenotypic susceptibility in the F_1 population indicates that the R allele is recessive. Furthermore, the genotype ratio in the F_2 population resulting from such a genetic cross will be RR: 2RS: SS and 75% or 25% of the population will be phenotypically resistant, if the R allele is dominant or recessive respectively.

Demonstration of dominance using mass matings is not necessarily straightforward if the parent populations do not consist of 100% homozygous individuals for the resistance-conferring mutation(s). The benzimidazole resistance genotypes of the parent MHco3 (male) and MHco4 (female) *H. contortus* recovered from the abomasum of the recipient lamb were determined by PCRs on individual DNA lysates, using a primer pair flanking the isotype 1 β tubulin codon 167 and 200 SNPs. The A/T SNPs at positions 167 and 200 of the isotype 1 β tubulin gene were determined by SNP analysis pyrosequencing. The F200Y genotypes of MHco3 *H. contortus* were determined at various times during the study using the same method. Correlation between the isotype 1 β tubulin codon 200 SNP and the thiabendazole resistance phenotype in the EHA was determined by comparing the F167Y and F2000Y genotypes of 25 MHco3 (ISE) *H. contortus* L_1 that had hatched in 0.1 μg/ml thiabendazole with those of 31 eggs that did not develop or hatch.

3.3 Results

3.3.1 Development of a method to enable successful recovery of the progeny of single *H. contortus* parents

Single male (MHco3) and single female (MHco4) *H. contortus* were surgically transplanted into the abomasa of worm free lambs (recipients A, B, C, D, E and F). Support for the successful recovery of progeny of the single parent crosses was first sought by monitoring the FWECs of the recipient ewes and showing their *H. contortus* identity using gross parasitological and molecular methods. Conclusive proof of a successful single parent genetic cross was sought by the molecular genetic analysis of F_2 progeny produced by interbreeding populations of F_1 nematodes.

3.3.1.1 Recovery of putative F_1 progeny of the single parent genetic cross

No complications or adverse effects were encountered during or following surgical transfer. Positive FWECs of between 1 egg per 10 g and 1.5 epg were identified at some stage between days 21 and 54 after donor infection (days 7 and 47 after surgical transplantation) in 5 of the 6 recipient lambs. These counts were sustained in two cases (recipient B and recipient D). The FWECs are shown in Fig 3.3.

Between 8 and 50 L_3 were recovered from all of the daily coprocultures that corresponded with positive FWECs. Sheathed parasitic L_3 were also recovered from some coprocultures that were started when FWECs were zero. DNA lysates were only made from L_3 that were considered at the time to be surplus to requirements for subsequent donor sheep infection. The presence of *H. contortus* was only confirmed in L_3 recovered from recipients A and D. The results of tests that were performed to identify the species and genus of these L_3 are shown in Table 3.1.

No nematodes were recovered from the abomasa of any of the recipient sheep after they were killed 60 days (recipients A, B and C) or 25 days (recipients D, E and F) after surgical transfer.

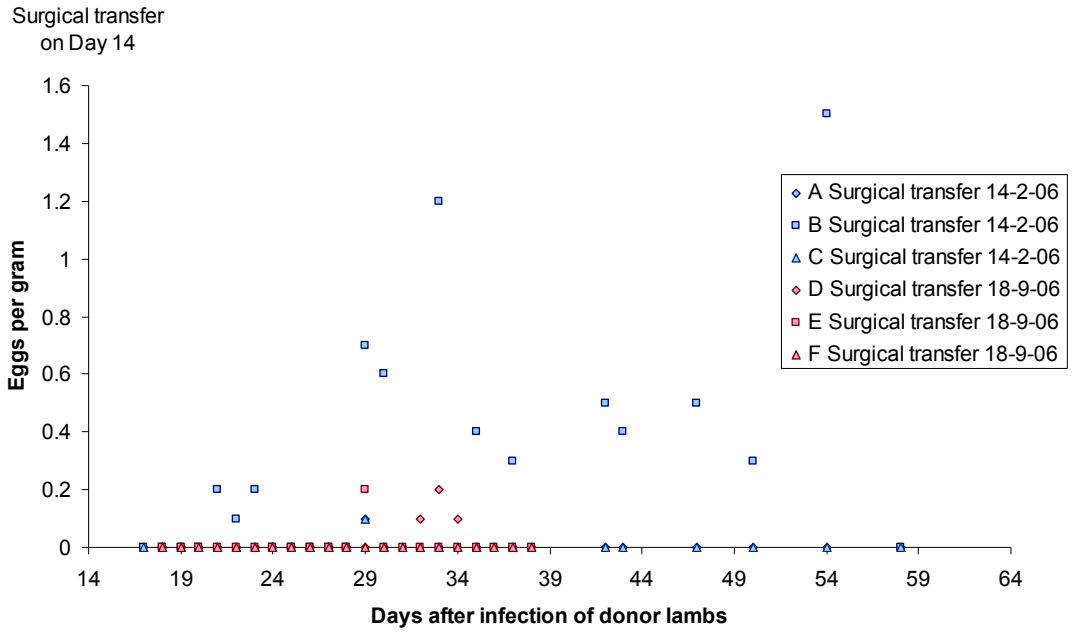
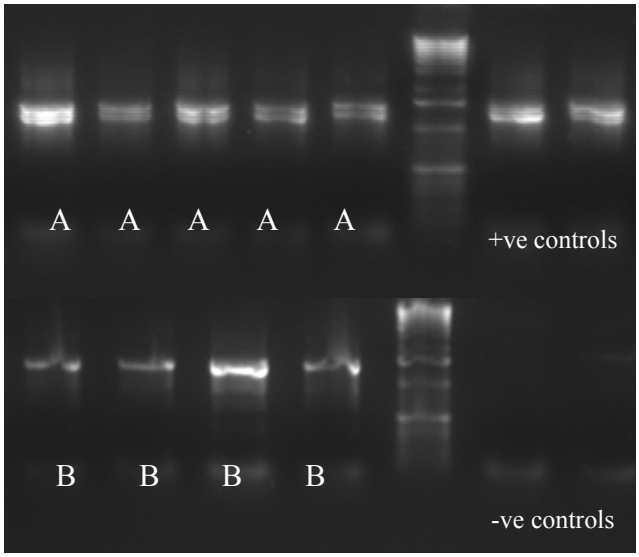


Fig 3.3: FWECs of the six recipient sheep following surgical transfer of single male and female parents on day 14.

Recipient	Number of individual L_3 lysates made	Lysates showing products of *Haemonchus* specific ITS2 PCR	Lysates showing products of NTS PCR (Fig 3.4)	L_3 morphology
A	42	8 (325 bp)	7 (~1.4 and 1.5 kb)	*Haemonchus* spp.
B	28	0	7 (~1.5 kb)	*Teladorsagia* spp.
C	8	1 (325 bp)	3 (~1.5 kb)	-
D	59	35 (325 bp)	36 (~1.4 and 1.5 kb)	*Haemonchus* spp. *Trichostrongylus* spp.
E	-	-	-	(*Strongyloides* spp. +++)
F	-	-	-	(*Strongyloides* spp. +++) *Nematodirus helvetianus*

Table 3.1: Morphological examination and molecular tests to identify putative F_1 progeny of single parent genetic crosses.



Double bands ~1.4/1.5 kb
(*H. contortus*)

Single band ~1.5 kb
(*T. circumcincta*)

Fig 3.4: NTS PCR products run on a 1% agarose gel (John Gilleard, *personal communication*) using lysates of individual putative F_1 progeny of single parent genetic crosses from recipients A and B and on positive *H. contortus* controls.

3.3.1.2 Infection of putative F_2 donor sheep

About 100 L_3 recovered from the coprocultures of recipient B were used to infect a worm-free donor lamb with the aim of producing an F_2 generation that could be phenotypically and genotypically characterised. (This was done, before there was time to confirm the species identity of the putative F_1 nematodes.) Coprocultures from this donor sheep only yielded short tailed L_3 and 17 (15 ♀ and 2 ♂) adult *T. circumcincta* were recovered from the abomasum after the sheep was killed, 63 days after infection. These were stored in absolute ethanol in case they may be of future interest.

Twenty eight medium sheathed tail length L_3 recovered from the coprocultures of recipient D were used to infect a worm-free donor lamb. The putative F_2 FWECs of this donor are shown in Fig 3.5.

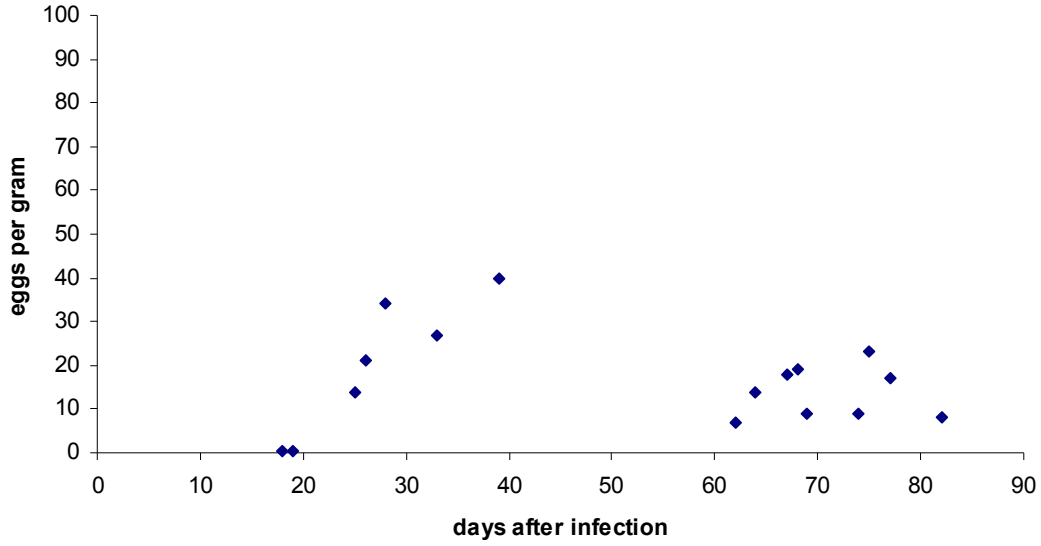


Fig 3.5: Faecal worm egg counts of a donor sheep infected with putative F_1 *H. contortus* progeny of a single parent genetic cross (recipient D) (Counts were performed on the days shown on the graph.)

More than 95% of the eggs shed by the putative F_2 donor were consistently shown to be those of *H. contortus* on the basis of egg morphometrics and fluorescence of the egg shells following staining with fluorescein isothiocyanate labeled peanut agglutinin. Morphological examination of L_3 recovered from coprocultures consistently showed 98% to be *Haemonchus* spp. and 2% to be *Trichostrongylus* spp.. Molecular proof of the *H. contortus* identity of individual L_3 was provided by the consistent identification of 325 bp or doublet ~1.4 and ~1.5 bp products of ITS2 or NTS region PCRs respectively. Three adult female *H. contortus* were recovered from the abomasum after the sheep was killed, 82 days after infection. These were stored in absolute ethanol in case they may be of future interest.

3.3.1.3 In vitro *bioassays*

EHAs and LFIAs were performed three times using the putative F_2 egg and L_1 progeny of the genetic cross from recipient D. The ED_{50}, LFI_{90} and LFI_{99} values obtained from these assays were compared to values obtained from contemporary donor sheep infected

with the parent MHco3 and MHco4 strains of *H. contortus* and with a *H. contortus* isolate that was obtained and characterised during the investigation of a concurrent outbreak of haemonchosis affecting sheep in fields adjacent to the animal housing and coproculture room at the Moredun Research Institute (Table 3.2). The putative F_2 phenotype was benzimidazole resistant (EHA ED_{50} ≥0.1 µg/ml TBZ) and similar to MHco4 (ivermectin resistant) and the field *H. contortus* isolate in the LFIA.

H. contortus strain	EHA ED_{50} (µg/ml TBZ) [±SD]	LFIA LFI_{90} (µg/ml IVM) [±SD]	LFIA LFI_{99} (µg/ml IVM) [±SD]
MHco3 (ISE)	0.054 [±0.013]	0.032 [±0.01]	0.048 [±0.014]
MHco4 (WRS)	0.18 [±0.086]	0.057 [±0.016]	0.095 [±0.033]
Putative MHco3/4.F_2	0.14 [±0.002]	0.051 [±0.021]	0.089 [±0.035]
Moredun field isolate	0.477	0.043 [±0.007]	0.082 [±0.024]

Table 3.2: ED_{50} values obtained from egg hatch assays and LFI_{90} and LFI_{99} values obtained from larval feeding inhibition assays performed using the putative F_2 progeny of the single parent genetic cross from recipient D.

3.3.1.4 Molecular genetic analysis of the attempted single parent genetic cross

3.3.1.4.1 Recipient A

H. contortus specific microsatellite PCRs yielded sufficient product to produce useable Genescan traces for 40 of 42 lysates prepared from the individual putative F_1 L_3 progeny obtained from recipient A. The allele frequencies at the four microsatellite loci are summarised in Table 3.3.

The frequencies of the two alleles of Hcms15 were approximately 50%, but those of Hcms27 and Hcms22co3 were inconsistent with the values of 25%, 50% or 75% that would have been predicted for the F_1 progeny of a single parent genetic cross. Furthermore, the frequencies of heterozygotes at these three loci were all inconsistent with the predicted results for the F_1 progeny of a single parent genetic cross. The analytical value of locus Hcms94 was limited by the fact that it was monomorphic.

Microsatellite locus	Allele	Number of alleles (%)	Number of heterozygotes (%)	Consistent with Mendelian allele frequency (±10%)	Consistent with Mendelian heteroygote frequency
Hcms15	272	33 (45.8%)	4 (11%)	✓	×
	287	39 (54.2%)			
Hcms27	338	8 (12.5%)	4 (12.5%)	×	×
	344	0			
	348	0			
	358	56 (87.5%)			
Hcms94	193	0	0	monomorphic	monomorphic
	143	0			
	233	64 (100%)			
Hcms22co3	234	4 (6.7%)	2 (6.7%)	×	×
	242	0			
	250	56 (93.3%)			
	258	0			

Table 3.3: Allele frequencies at four microsatellite loci of the *H. contortus* F_1 progeny of a single parent genetic cross from recipient A.

The allele frequencies of the putative L_3 progeny of recipient A for the four microsatellite loci were compared with single L_3 lysate data for MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus* (Fig 3.6). The data show that the putative L_3 progeny of the genetic cross have similar allele frequencies to a population of the parental MHco3 (ISE) strain of *H. contortus*, while being very different from the MHco4 (WRS) and MHco10 (CAVR) strains.

3.3.1.4.2 Recipient B

PCR amplifications of *H. contortus* microsatellite loci using DNA lysate template prepared from 8 individual L_3 putative progeny of recipient B were unsuccessful (it was subsequently shown that these lysates were probably made from *T. circumcincta*, and not *H. contortus*).

Microsatellite locus	Allele	Putative F_1 nematodes	MHco3 *H. contortus*	MHco4 *H. contortus*	MHco10 *H. contortus*
Hcms15	272	45.8%	45%	0	0
	287	54.2%	55%	100%	100%
Hcms27	338	12.5%	3.7%	41.3%	52.3%
	344	0	0	21.7%	35.7%
	348	0	0	4.4%	0
	358	87.5%	96.3%	32.6%	12%
Hcms94	172	0	0	11.5%	no data
	174	0	0	13.5%	
	177	0	0	3.8%	
	193	0	3.3%	1.9%	
	194	0	0	1.9%	
	196	0	0	15.4%	
	201	0	0	3.9%	
	225	0	1.7%	0	
	231	0	0	3.9	
	233	100%	95%	26.9%	
	235	0	0	13.5%	
Hcms22co3	234	6.7%	7.3%	29.8%	0
	242	0	0	34%	0
	250	93.3%	92.7%	19.9%	86.5%
	258	0	0	16.2%	13.5%

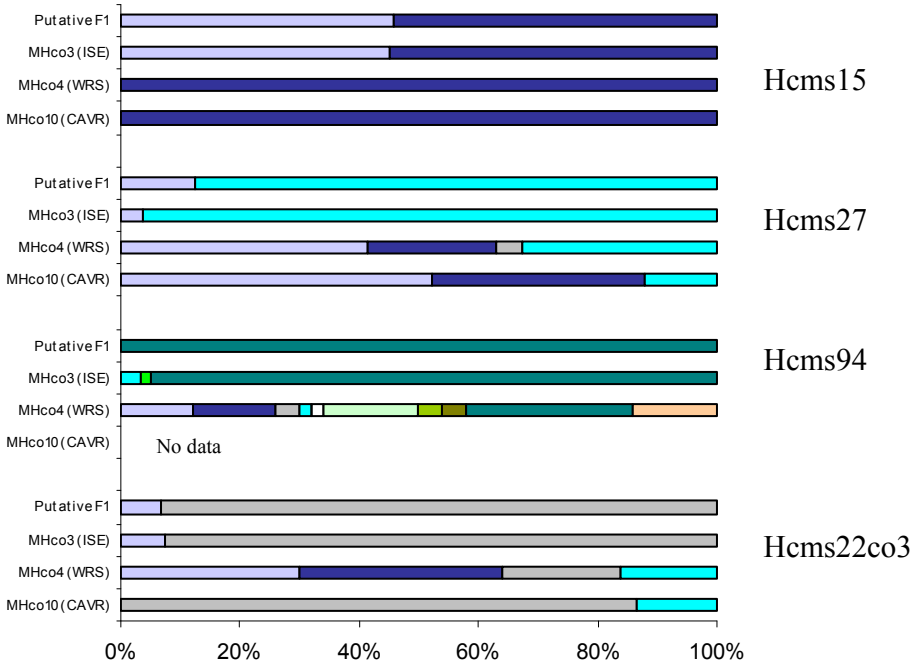


Fig 3.6: Comparison of microsatellite loci allele frequencies between *H. contortus* F_1 progeny obtained from recipient A and strains of *H. contortus* concurrently maintained at the Moredun Research Institute.

3.3.1.4.3 Recipient C

PCRs using DNA lysate template prepared from 8 individual L_3 putative progeny of recipient C only yielded sufficient product to generate useable Genescan traces for 10 alleles at the four loci in five nematodes, so insufficient data were available for genetic analysis.

3.3.1.4.4 Recipient D

H. contortus specific microsatellite PCRs yielded sufficient product to produce useable Genescan traces for 39 lysates prepared from the individual putative F_2 L_3 progeny of recipient D. The isotype 1 β tubulin codon 200 genotypes of 27 F_2 L_3 were determined by PCR and pyrosequencing. The allele frequencies at these marker loci are summarised in Table 3.4 and can be seen to be inconsistent with simple Mendelian ratios predicted for an F_2 generation from a single pair mating. Indeed the presence of 5 alleles at locus 8a20 alone indicates the population is highly unlikely to be the result of a single pair mating. A maximum of four alleles should be seen unless there was a *de novo* mutation arising in the parental or F_1 germ lines, or the marker is inherently unreliable.

The allele frequencies of the putative F_2 L_3 progeny of recipient D for the three microsatellite loci were compared with known allele frequencies in MHco3 (ISE), MHco4 (WRS) and Mco10 (CAVR) strains of *H. contortus* (Libby Redman, *data on file*). The isotype 1 β tubulin codon 200 SNP genotype frequencies were also compared with data for individual MHco3 (ISE) and MHco4 (WRS) *H. contortus* lysates gathered throughout this study. Fig 3.7 shows that the putative L_3 F_2 progeny of the genetic cross were similar to (but not the same as) a population of MHco3 (ISE) *H. contortus*, while being very different from the MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus*.

Marker locus	Allele	Number of alleles (%)	Consistent with Mendelian allele frequency (±10%)
8a20	192	12 (18.8%)	×
	196	7 (10.9%)	
	208		
	212		
	214		
	220		
	224		
	232	11 (17.2%)	
	240	30 (46.9%)	
	244		
	248	4 (6.2%)	
Hcms36	148	26 (41.9%)	×
	150		
	152	36 (58.1%)	
	154		
Hcms22co3	234	7 (14.6%)	×
	242		
	248		
	250	41 (85.4%)	
	254		
	258		
β tubulin 200	A	7 (13%)	×
	T	47 (87%)	

Table 3.4: Allele frequencies at four marker loci of the *H. contortus* F_2 progeny of a single parent genetic cross from recipient D.

The bulk microsatellite Genescan traces the putative F_2 L_3 progeny of recipient D were very all clearly different from those for MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus*, which were maintained in donor sheep kept in pens adjacent to the genetic cross recipient and donor sheep and using the same coproculture room. The bulk microsatellite Genescan traces were similar to those for the MHco3 strain of *H. contortus* and for a *H. contortus* isolate that was obtained and characterised during the investigation of a concurrent outbreak of haemonchosis affecting sheep in fields adjacent to the animal housing and coproculture room at the Moredun Research Institute (Fig 3.8).

Marker locus	Allele	Putative F_2 nematodes	MHco3 (ISE) *H. contortus*	MHco4 (WRS) *H. contortus*	MHco10 (CAVR) *H. contortus*
Hcms8a20	192	18.8%	12.5%		
	196	10.9%	18.7%	1.7%	
	208				2.5%
	212				2.5%
	220				1.2%
	224				1.2%
	232	17.2%	29.2%	3.3%	
	240	46.9%	39.6%	53.3%	46.3%
	244			11.7%	
	248	6.2%		30%	46.3%
Hcms36	148	41.9%	53.3%	15%	22.2%
	150			10%	25.9%
	152	58.1%	46.7%	63.3%	13%
	154			11.7%	38.9%
Hcms22co3	234	14.6%	8.3%	32.6%	
	242			16.3%	9%
	248			4.6%	
	250	85.4%	91.7%	41.9%	65.4%
	254			4.6%	
	258				25.6%
β tubulin 200	A	13%	5%	25%	no data
	T	87%	95%	75%	

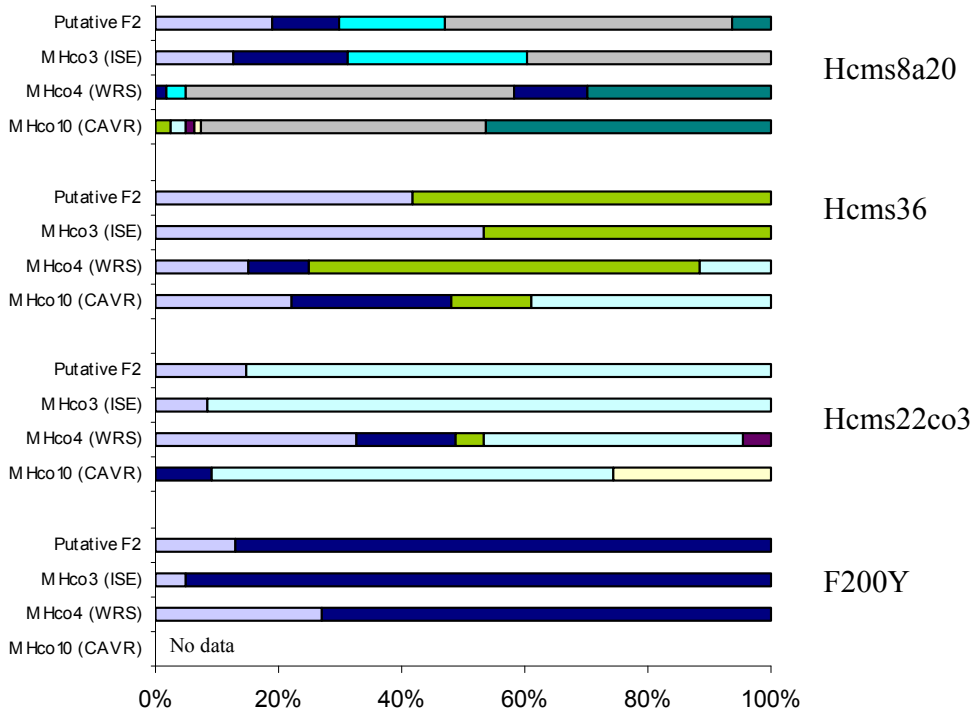


Fig 3.7: Allele frequencies at microsatellite loci Hcms8a20, Hcms36 and Hcms22co3 and at the isotype 1 β tubulin codon 200 SNP locus.

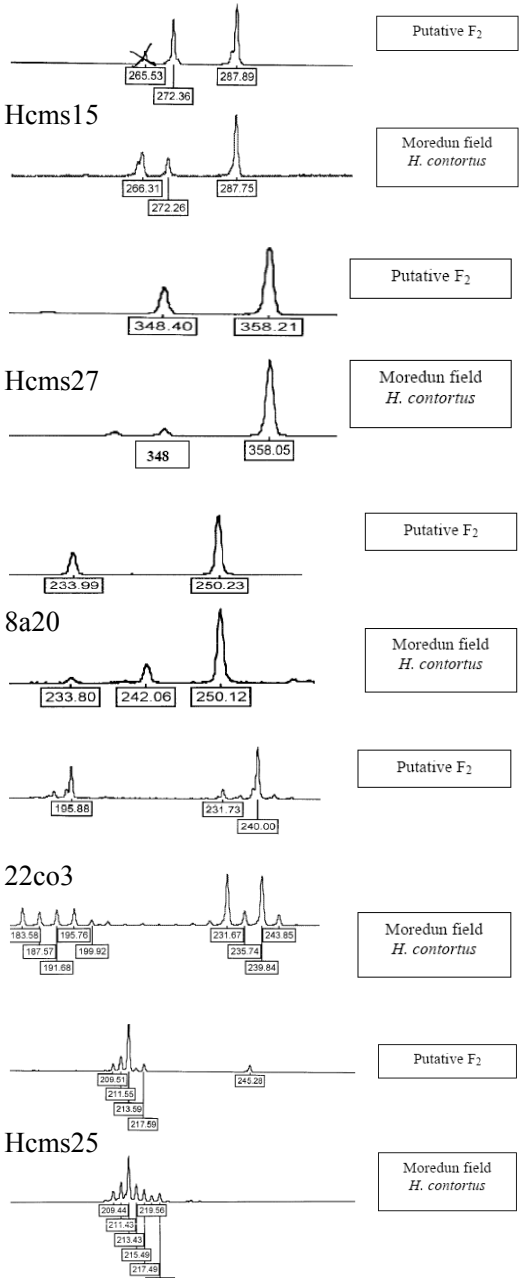


Fig 3.8: Bulk microsatellite Genescan traces comparing the putative F_2 *H. contortus* with a neighbouring field isolate derived from a concurrent disease outbreak. (The bulk genotyping approach is not a standard technique and has only been used to enable subjective comparisons between populations. Areas under larger peaks approximate allele frequencies, while the origin of smaller peaks is less clearly defined.)

3.3.2 Reproductive biology and mating patterns of *H. contortus*

3.3.2.1 The duration of fertilised egg shedding after removal of males

Two hundred adult female MHco3 (ISE) *H. contortus* were surgically transferred into the abomasum of a worm free recipient lamb. Subsequent egg shedding and development were monitored to determine the duration of fertilised egg shedding after removal of males. Once fertilised egg shedding ceased, the recipient lamb was orally infected with a morphologically different MHco10 (CAVR) strain of *H. contortus* to ascertain if female MHco3(ISE) *H. contortus* would rebreed following a period of absence from males.

Fertile *H. contortus* eggs were seen in faecal samples within 24 hours of the surgical transfer of 200 adult female nematodes. The FWEC rose to a maximum of 310 epg (equivalent to about 4,000 eggs per surviving female *H. contortus* per day) on day 27 (4 days after surgical transfer), before falling to about 13 (SD ± 6.4) epg between days 43 and 51 after the infection (20 and 28 days after surgical transfer) of the initial donor lamb with MHco3 (ISE) *H. contortus* L_3. The FWECs then increased again from day 55 after donor infection, corresponding to 14 days after oral infection with MHco10 (CAVR) *H. contortus* L_3.

A mean of 75% (SD ± 19.7%) of the eggs that were shed over the first five days after the surgical transfer of adult female *H. contortus* hatched. The mean percentage of eggs hatching then fell to about 1% by 9 days after surgical transfer of adult female *H. contortus* and then remained at 0.2% for a further 5 days. No eggs subsequently hatched until about day 60, 20 days after oral infection with MHco10 (CAVR) *H. contortus* L_3. The FWECs and the corresponding percentage of eggs hatching are shown in Fig 3.9.

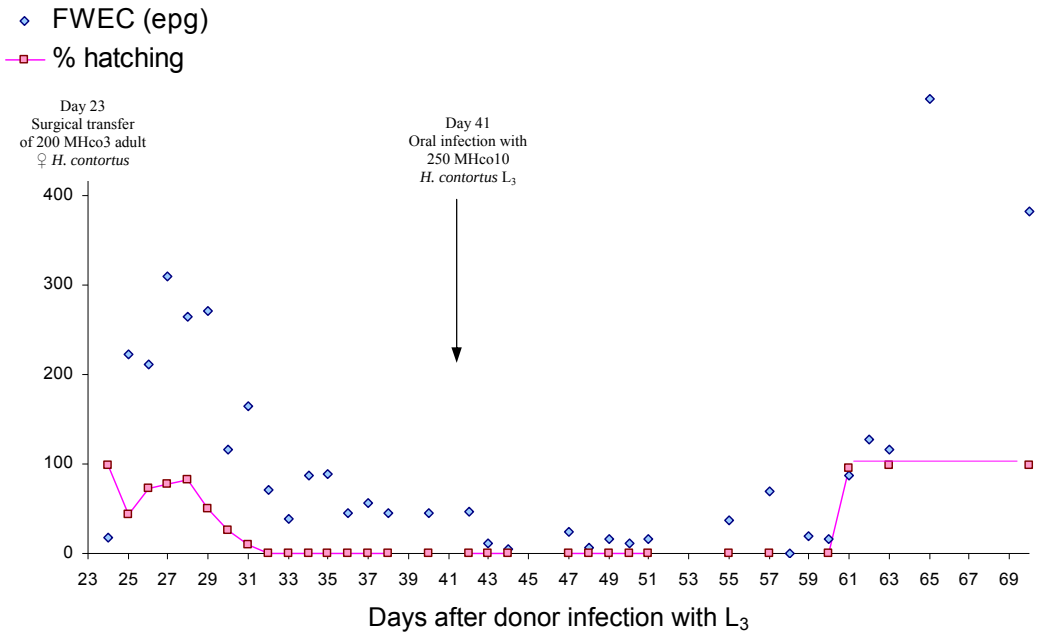


Fig 3.9: FWECs and mean percentage of hatching of eggs in two aliquots extracted from corresponding faeces, first following the surgical transfer of adult MHco3 (ISE) *H. contortus* and then following oral infection with MHco10 (CAVR) *H. contortus* L_3.

Twenty seven adult male, 39 adult female *H. contortus* without vulval flaps (these would have been predominantly MHco10, although it is possible that a small number of MHco3 females had a similar smooth vulval flap morphology) and 28 adult female *H. contortus* with vulval flaps (predominantly MHco3) (Fig 3.10) were recovered at post-mortem from the abomasum after the lamb was euthanased on day 70. The probable MHco10 (CAVR) *H. contortus* females shed twice as many eggs as the MHco3 (ISE) *H. contortus* females within 4 hours of transfer individuals to RPMI in the wells of 24 well plates and incubation at 37°C in 5% CO_2. Means of 62.5% (SD ± 22.1%) and 44.7% (SD ± 32.2%) respectively of the eggs shed by the MHco10 (CAVR) and MHco3 (ISE) *H. contortus* females hatched. The mean (±SEM) numbers of eggs shed and numbers of L_1 hatched from individual *H. contortus* with vulval flaps (predominantly MHco3) and without vulval flaps (predominantly MHco10) are shown in Fig 3.11.

Fig 3.10: An MHco3 (ISE) female showing the linguiform vulval flap morphology. B: Displacement of the vulval flap during mating.

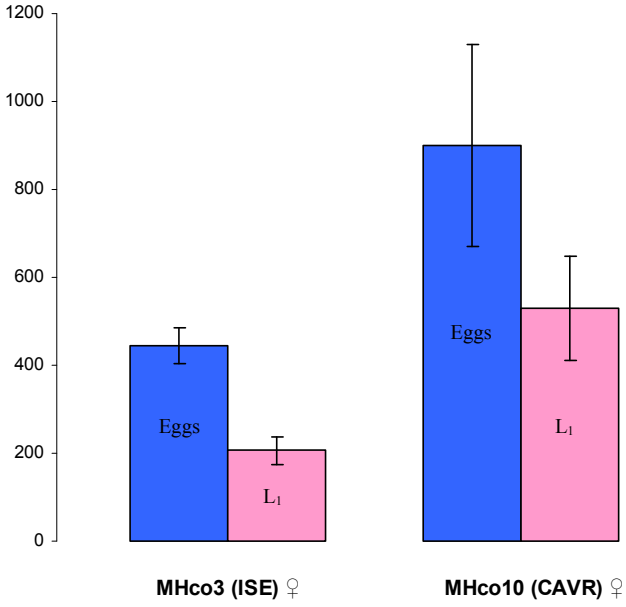


Fig 3.11: Mean egg shedding and L_1 hatching from individual adult female *H. contortus* with (predominantly MHco3) and without (predominantly MHco10) vulval flaps.

3.3.2.2 Egg laying by day 14 female H. contortus

Day 14 late L_4/immature adult female MHco3 *H. contortus* were surgically transferred into the abomasa of two worm free lambs (recipients X and Y). FWECs of the recipient lambs were monitored daily and eggs were extracted and incubated in tapwater to

monitor development and hatching. PCRs were performed on lysates prepared from individual eggs using primers flanking a panel of microsatellite markers to determine heterozygosity.

Trichostrongyle eggs were voided in the faeces of both recipient lambs that had been surgically transplanted with day 14 female *H. contortus* from 2 days after surgical transfer (day 16 after infection of the donor lambs with L_3). A mean of 106 (SD ±89) epg were voided daily in the faeces of recipient X between days 21 and 33, while a mean of 41 (SD ±26) epg was voided daily in the faeces of recipient Y between days 21 and 93 (Fig 3.12). These FWECs equated to about 100,000 and 40,000 eggs shed daily in the faeces of recipients X and Y respectively. One hundred and eight and 52 adult female only *H. contortus* were recovered postmortem from the abomasa of recipients X and Y on days 44 and 99 following initial L_3 infection of donor lambs. Thus, each unfertilised adult female *H. contortus* shed about 1,000 eggs per day. The egg counts of recipient X were higher than those of recipient Y, probably associated with the higher number of female *H. contortus* that were transplanted and higher numbers recovered postmortem from the abomasum. None of the eggs voided by either recipient X or recipient Y were identified as hatching following incubation at 24^{o}C, or at a range of other temperatures. However, about 50% of the eggs voided by each recipient lamb developed to a multi-cell, blastula or undifferentiated gastrula stage within a few hours of extraction from faeces. The remaining eggs contained a morula or one or two degenerate cells (Fig 3.13). When examined under differential interface contrast microscopy, many of these eggs appeared to be thin-shelled and morphologically abnormal (Fig 3.14). Ninety-five percent of the unfertilised eggs shed by the unmated female *H. contortus* stained positively with fluorescent peanut agglutinin. No further development was observed following 24 or 48 hours incubation.

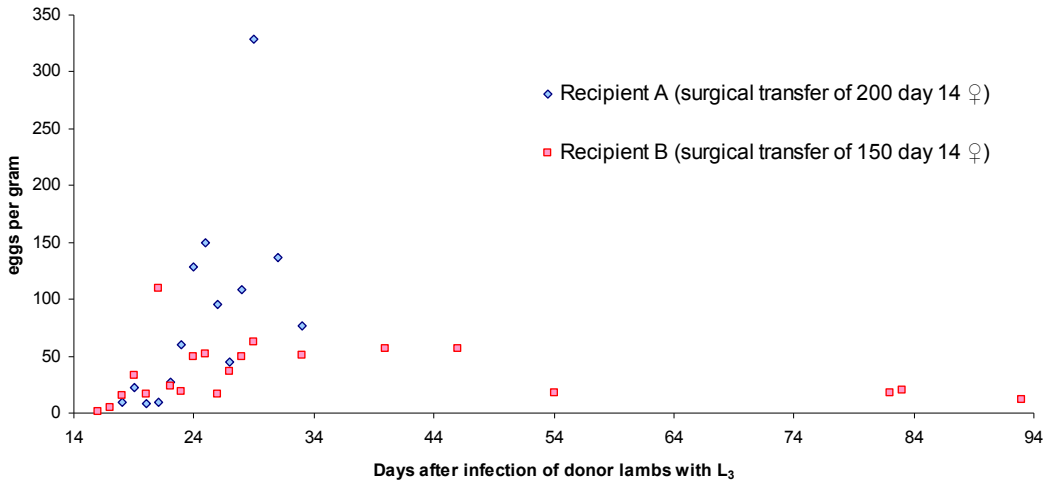


Fig 3.12: FWECs of two recipient lambs following the surgical transfer of day 14 immature female *H. contortus.* (The egg counts of Recipient X were higher than those of recipient Y, probably because of the higher number of female *H. contortus* that were transplanted and higher numbers recovered postmortem from the abomasum of the recipient lamb.)

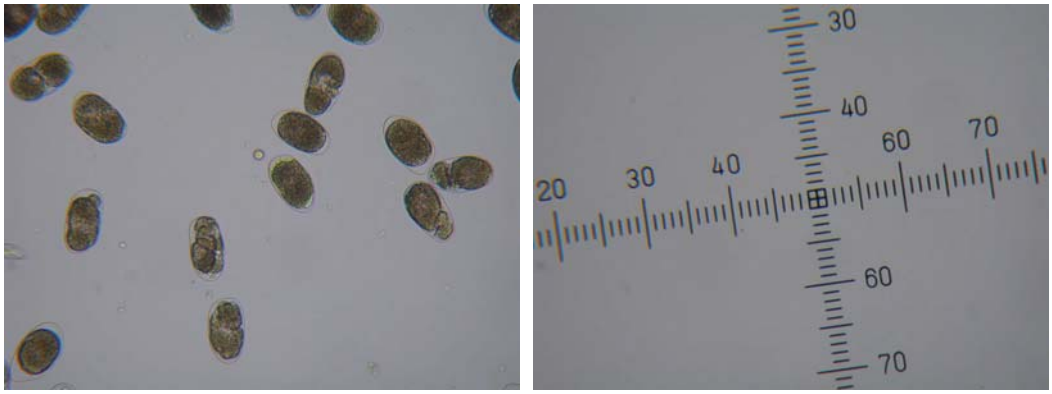


Fig 3.13: Normal sized (~80 µm long), thin walled eggs shed by unfertilised female *H. contortus* from recipient X examined under standard light microscopy (incubation for 48 hours at 22°C). Some contain a single cell, some have arrested development at a two cell stage, and some have continued to develop to a 4 or 5 cell stage.

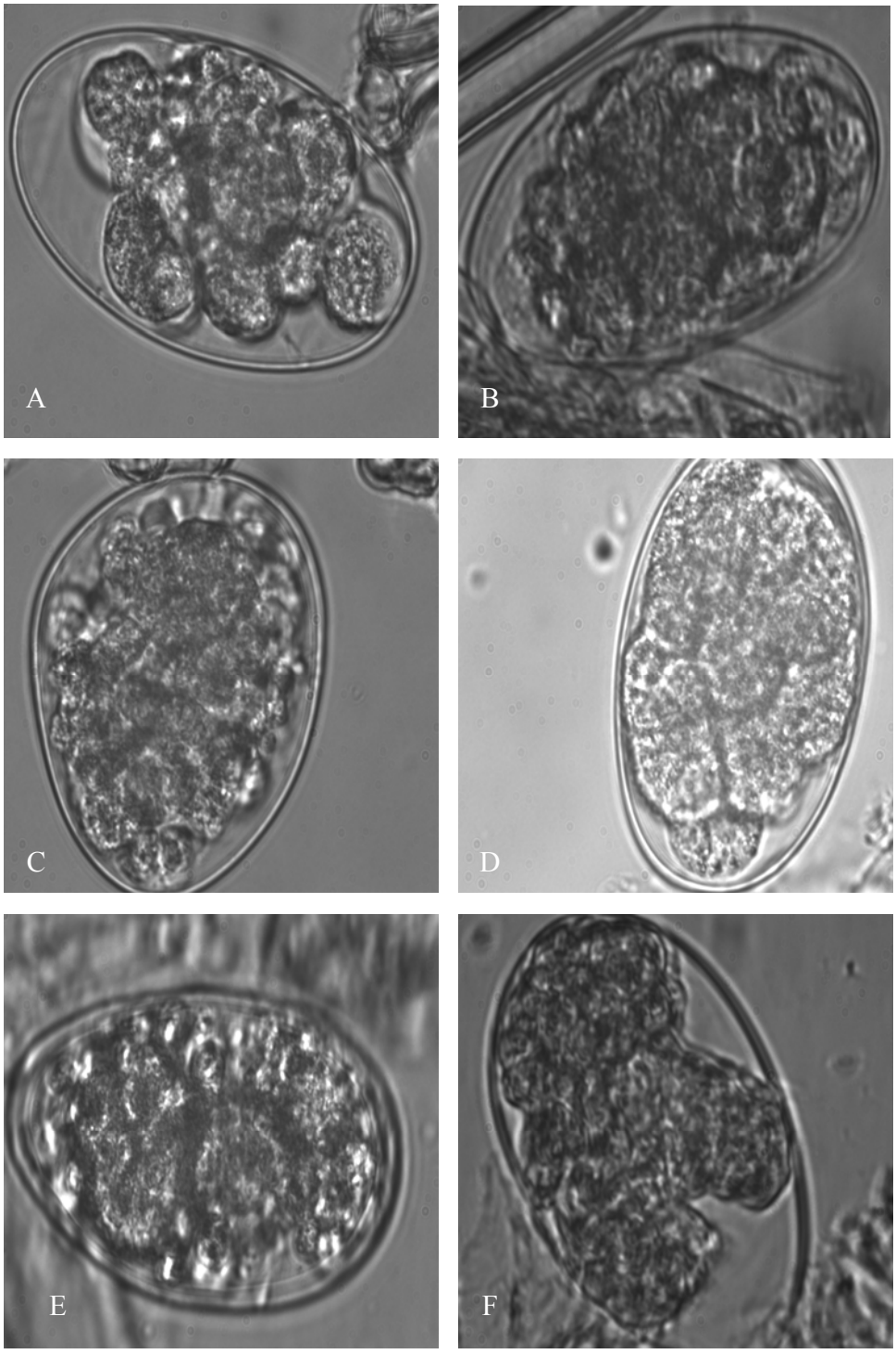


Fig 3.14: Differential interface contrast microscopy of unfertilised eggs shed by recipient Y, showing abnormal morphology of blastulae (A, B and C) or gastrulae (D, E and F) in eggs shed by unfertilised female *H. contortus*. The thin shells have given rise to abnormally rounded shapes. (Incubation for 48 hours at 22^{o}C.)

Microsatellite markers were amplified from DNA template prepared from 30 of 56 unfertilised eggs that had been shed by female *H. contortus* which had been surgically transferred as day 14 late L_4/immature adults into the abomasum of recipient X. (PCRs for two 'new' markers [40506 and 59736] did not lead to the generation of useful Genescan traces.) Fifteen of 30 unfertilised egg lysates were heterozygous at one or more of three useable microsatellite loci, while 5 were heterozygous at two loci. The percentage of heterozygotes was highest at the most polymorphic locus (Hcms25). The percentages of monomorphic and heterozygous eggs at microsatellite loci Hcms25, Hcms36 and 3561 are shown in Fig 3.15.

Monomorphic (n)	19	12	14
Heterozygous (n)	11	4	2
PCR failure	0	14	14

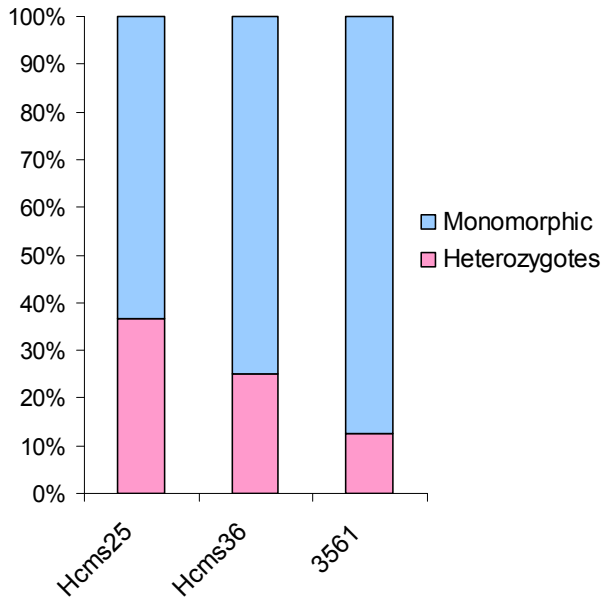


Fig 3.15: Percentage of monomorphic and heterozygous embryos at three microsatellite loci in a population of 30 unfertilised eggs shed by female *H. contortus* that had been surgically transferred as day 14 late L_4/immature adults (recipient X).

Microsatellite markers were also amplified from the DNA template of 86 unfertilised eggs that had been shed by female *H. contortus* which had been surgically transferred as day 14 late L_4/immature adults into the abomasum of recipient Y. Locus Hcms27 was monomorphic throughout the MHco3 (ISE) isolate, and therefore of no value for the determination of heterozygosity. 42 of the 86 unfertilised eggs were heterozygous at between 1 and 6 of the 10 polymorphic loci. The percentage of heterozygous eggs at each autosomal locus was related to the level of polymorphism (and probably confounded by null alleles). None of the unfertilised eggs were heterozygous at loci 18210 or Hcms22co3. Eighteen of the unfertilised eggs were heterozygous at between 1 and 3 of the 3 X chromosome loci. The percentages of monomorphic and heterozygous eggs at the 7 polymorphic autosomal microsatellite loci and at the three polymorphic X chromosome loci are shown in Fig 3.16. PCRs that failed to amplify the microsatellite markers, probably arose due to poor DNA lysate template quality or primer degradation, rather than due to true null alleles are shown. The microsatellite allele data are shown in appendix 3.1.

Monomorphic (n)	43	50	50	44	49	59	56	64	53	56
Heterozygous (n)	16	17	14	7	4	0	0	7	9	7
PCR failure	27	19	22	35	33	27	31	15	24	23

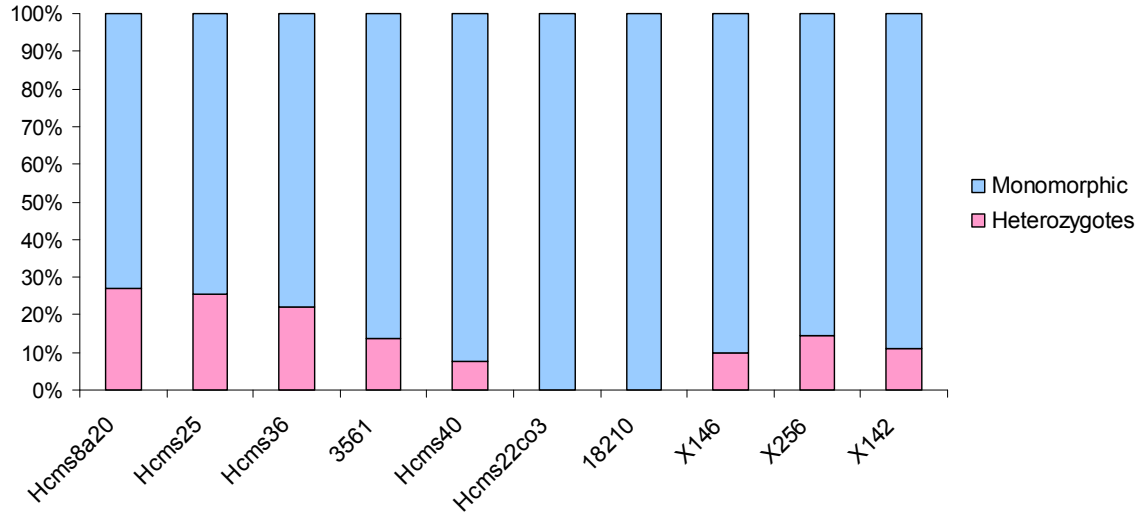


Fig 3.16: Percentage of homozygotes and heterozygotes at seven autosomal and three X chromosome microsatellite loci in a population of 86 unfertilised eggs shed by female *H. contortus* that had been surgically transferred as day 14 late L_4/immature adults (Recipient Y).

The observed heterozygosities (H_o) at 10 polymorphic loci were lower than unbiased estimates of heterozygosity that would have been expected if all of the eggs were diploid (H_e) based on the frequencies of the alleles that were present (Table 3.5). Overall, these results suggest that some of the eggs shed by the unfertilised female *H. contortus* were diploid or polyploid, and a proportion may have been haploid.

	N	H_e	H_o	P-value	A
Hcms25	63	0.777	0.285	<0.001	6
Hcms36	63	0.489	0.222	<0.001	2
Hcms40	53	0.483	0.075	<0.001	2
22co3	59	0.327	0	<0.001	2
8a20	57	0.676	0.281	<0.001	5
18210	53	0.073	0	<0.001	2
3561	49	0.594	0.143	<0.001	3
X146	67	0.211	0.09	<0.001	2
X256	61	0.448	0.158	<0.001	3
X142	63	0.579	0.111	<0.001	3
All loci		**0.466***	**0.137**		

N: number of individuals genotyped
H_e: expected heterozygosity
H_o: observed heterozygosity
F_{is}: inbreeding coefficient
A: number of alleles

Table 3.5: Observed heterozygosities of unfertilized eggs at 7 microsatellite loci and expected heterozygosity if the eggs had been fertilised and diploid. (* Comparison of the H_0 value with this H_e value, calculated from the alleles present in the unfertilised eggs may not be as meaningful as comparison with the H_e value for a population of MHco3(ISE) *H. contortus*, because H_o will generally be lower than H_e due to the presence of null alleles.)

Thirty-five of 48 unmated adult female *H. contortus* shed a mean of about 400 (±450 SD) unfertilised eggs within 6 hours of transfer to the wells of 24 well plates. The cells within a small proportion of these eggs developed to morulae.

3.3.2.3 Studies of interbreeding between different strains of H. contortus

A series of co-infection experiments using mixtures of two different *H. contortus* strains were undertaken in order to test the efficiency of mating between different isolates. FWECs of donor lambs were monitored in order to identify any differences in prepatent periods or egg shedding between the different parent strains of *H. contortus*. The

efficiency of mating between different strains of *H. contortus* was assessed by principal coordinate analysis for individual progeny using a panel of microsatellite markers.

The patterns of faecal worm egg production differed between the three individual comparable primary donor lambs infected at the same time with the same dose of different strains of *H. contortus*. For example, the rate of increase in FWEC from day 17 was greatest for the MHco3 (ISE) *H. contortus*, intermediate for the MHco10 (CAVR) *H. contortus* and lowest in MHco4 (WRS) *H. contortus*. The MHco10 (CAVR) *H. contortus* counts continued to increase until day 77, while those of the MHco3 (ISE) and MHco4 (WRS) *H. contortus* rose to maximum levels around day 30 before falling and remaining more-or-less constant until day 77. The FWECs of the three primary donor lambs are shown in Fig 3.17.

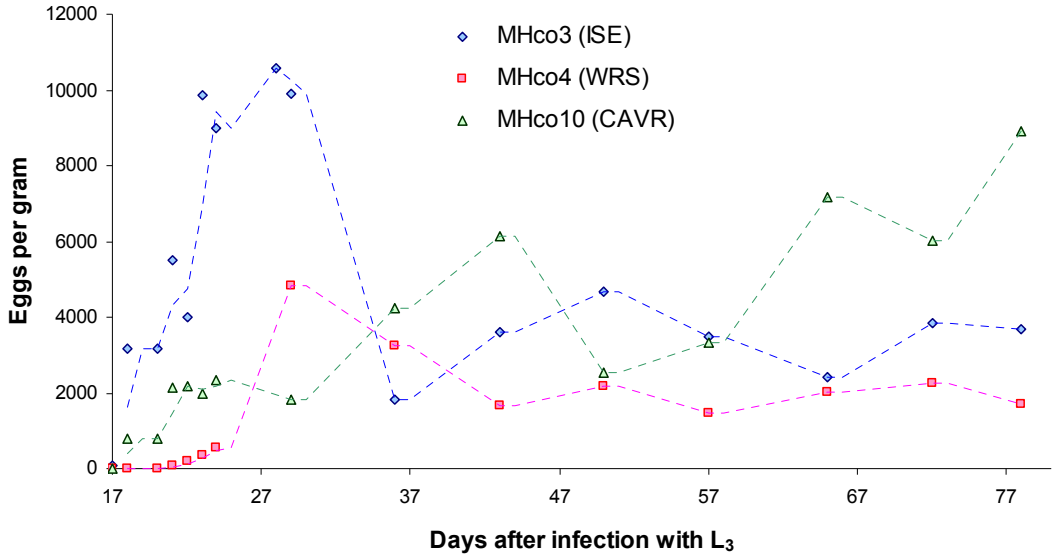


Fig 3.17: FWECs of donor lambs infected with different strains of *H. contortus*. (Note: While these patterns are clearly not statistically significant, because only one donor was required for each isolate, they are nevertheless an honest representation of the egg shedding by the animals that used in this study as donors for the populations of different isolates of *H. contortus* that were then used for the co-infection and interbreeding studies. As such, they are a valid basis for discussion. No attempt is made at any point in this thesis to imply that differences in the patterns seen can be statistically validated. The use of 6 animals per group with the aim of producing statistically valid representations of patterns of egg shedding (Georg

Von Samson-Himmelstjerna, *examiner's comment*) would have been unnecessary, prohibitively expensive and unethical within the context of this study. In fact, differences in the patterns of FWECs were observed following infection of donor lambs with the different strains of *H. contortus* throughout the course of study, for example Fig 3.27, but it is none-the-less acknowledged that further information based on egg shedding by large numbers of donor sheep would be valid in the interpretation of the results of interbreeding between different isolates.)

Between days 17 and 29, the patterns of FWECs of the three donor lambs infected with 4,000 L_3 of each of two *H. contortus* strains were similar to those predicted from the mean daily FWECs from the two primary donor lambs infected with the relevant *H. contortus* strains (Fig 3.18). The day 27 FWECs were highest in the two co-infections where the MHco3 (ISE) strain of *H. contortus* was present. Thereafter, the daily FWECs became erratic, coinciding with the onset of signs of lethargy and pallor in all three donor lambs.

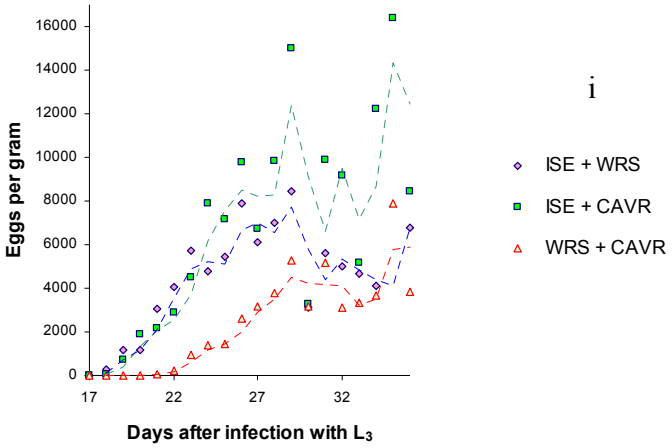


Fig 3.18: i) Actual FWECs of donor sheep infected with 4,000 MHco3 (ISE) and 4,000 MHco4 (WRS) *H. contortus* L_3, 4,000 MHco3 (ISE) and 4,000 MHco10 (CAVR) *H. contortus* L_3, or 4,000 MHco4 (WRS) and 4,000 MHco10 (CAVR) *H. contortus* L_3 on day 1.

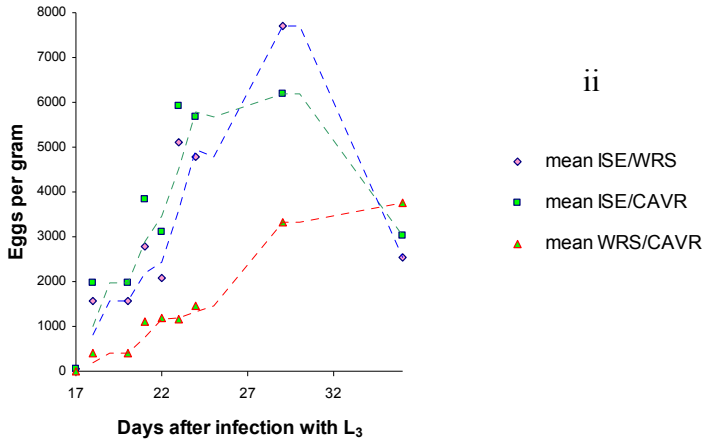


Fig 3.18: ii) Average FWECs of the primary MHco3 (ISE)/MHco4 (WRS) *H. contortus* L_3, MHco3 (ISE)/MHco10 (CAVR) *H. contortus* L_3 and MHco4 (WRS)/MHco10 (CAVR) *H. contortus* L_3 donors (Fig 3.17), infected on day 1.

The three donor lambs were euthanased on day 37, because of concern that they had become anaemic. The packed cell volumes, measured in blood samples collected into EDTA vacutainers, confirmed that all three lambs were anaemic (Table 3.6).

Donor lamb	PCV (%)
MHco3 (ISE) and MHco4 (WRS)	18
MHco3 (ISE) and MHco10 (CAVR)	28
MHco4 (WRS) and MHco10 (CAVR)	18
Normal range	25 - 39

Table 3.6: Day 37 packed cell volumes of three 6 month-old donor lambs infected with 8,000 *H. contortus* L_3 on day 1

Individual larvae were genotyped at multiple microsatellite loci and their multi-locus genotypes plotted by principal coordinate analysis (PCA). This provides a way of visualising the genetic identity of individual worms based on their multi-locus genotypes. Individual points on the PCA scatter plots represent a single worm and the closer two points are together the more similar their multilocus genotypes. The output shows information form three co-ordinates. When the cumulative variation in the data is largely explained by two co-ordinates, then the analysis is valid. The plots can be used to compare the similarity of different worm populations or laboratory strains. If there is

little genetic differentiation between the strains then the individual worm multilocus genotypes are randomly scattered. If on the other hand, the populations, or strains, are genetically distinct, then individual worm genotypes would tend to cluster on a population basis. Previous work using this type of analysis has shown that there is significant genetic differentiation between the MHco3 (ISE), MHco4 (WRS) and MHco10(CAVR) strains of *H. contortus* (Redman and others, 2008).

Twenty-two, 11, and 13 individual L_3 recovered, respectively, from the donor sheep infected with the MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus* were genotyped with 6 microsatellite markers (8a20, Hcms25, Hcms27, Hcms33, Hcms40, 22co3) (the microsatellite alleles are shown in appendix 3.2) and PCA plots of multi-locus genotypes were generated. These were the same markers previously used to genotype these strains generated from different donor sheep (Redman and others, 2008). A similar result was obtained as previously with significant genetic differentiation being found between the strains, with the MHco3 (ISE) and MHco10 (CAVR) *H. contortus* populations being the most divergent from each other and the MHco4 (WRS) strain being intermediate between the two (Fig 3.19). Because the parental strains can be genetically distinguished by PCA plots of multilocus genotype data, this approach can be used to study interbreeding of isolates in mixed infections.

Since this is a novel approach to studying interbreeding during co-infections, some explanation of the concept is necessary before presentation of the results. When donor sheep are infected with mixtures of two separate strains there are several possible outcomes depending on the extent to which the two strains interbreed. *Scenario 1*: the efficiency of breeding between worms of the two different strains is as high as it is between worms within each individual strain (the two different strains freely interbreed with no biological or reproductive barriers). If this is the case, the F_1 progeny harvested from the faeces of a co-infection should be an equal mixture of those that result from intra-strain matings and those that result from inter-strain matings. Therefore, PCA plots of multilocus genotypes of the F_1 progeny should consist of a mixture of points that

sit in the same space as the two parental clusters (F_1 progeny of intra-strain matings) and points that sit between the two parental clusters (F_1 progeny of inter-strain matings). *Scenario 2*: there is little or no inter-strain mating but the two strains develop and reproduce equally well during the co-infections. In this case, PCA plots of the F_1 generation would consist entirely of the progeny of intra-strain matings. Therefore PCA plots of multilocus genotypes of the F_1 progeny should consist entirely of data points that sit within the two parental clusters. *Scenario 3*: one of the strains dramatically out-competes the other in a mixed infection. In this case PCA plots of multilocus genotypes of the F_1 progeny should consist predominantly of data points that sit only within the cluster occupied by one of the parental strains.

The PCA plots for the eggs (F_1 progeny) harvested from donor lambs infected with equal numbers of L_3 of either the MHco3 (ISE) and MHco4 (WRS), or the MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus* broadly overlie those for the primary parental strains, with intermediate genotypes in between (Fig 3.20 and Fig 3.21) (the microsatellite allele data are shown in appendix 3.2). This is consistent with *scenario 1* and suggests that these pairs of strains interbreed efficiently during mixed infections. The results of the co-infection of MHco3 (ISE) and MHco10 (CAVR) are somewhat different. The PCA plots for the eggs (F_1 progeny) harvested from donor lambs infected with equal numbers of L_3 of either the MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* overlie those for the parental MHco3 (ISE), but not for the MHco10 (CAVR) parental strains. This is consistent with *scenario 3* with the MHco3 (ISE) isolate outcompeting the MHco10 (CAVR) isolate. There is some evidence of interbreeding between the MHco3 (ISE) and MHco10 (CAVR) as there are a few multilocus genotypes that are present between the two parental clusters (Fig 3.22).

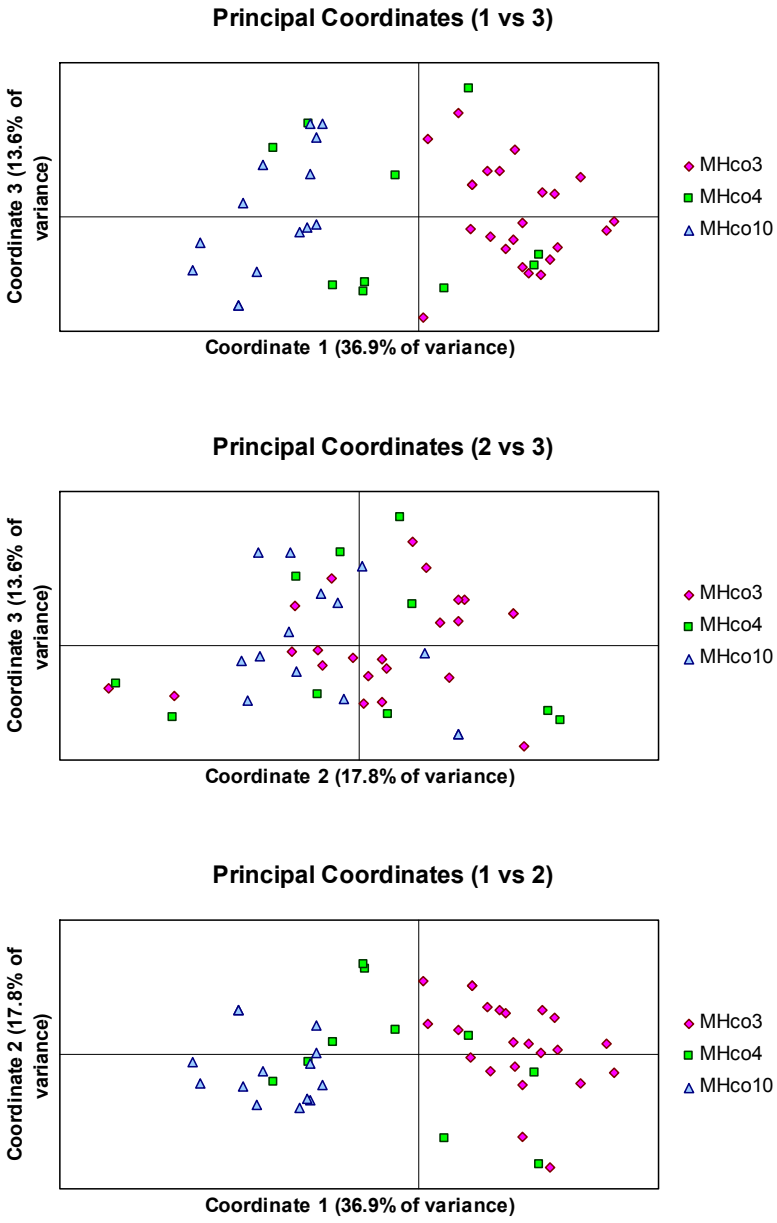


Fig 3.19: Principal coordinates analysis plots for MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* using a panel of 6 microsatellite loci. Each point represents an individual nematode.

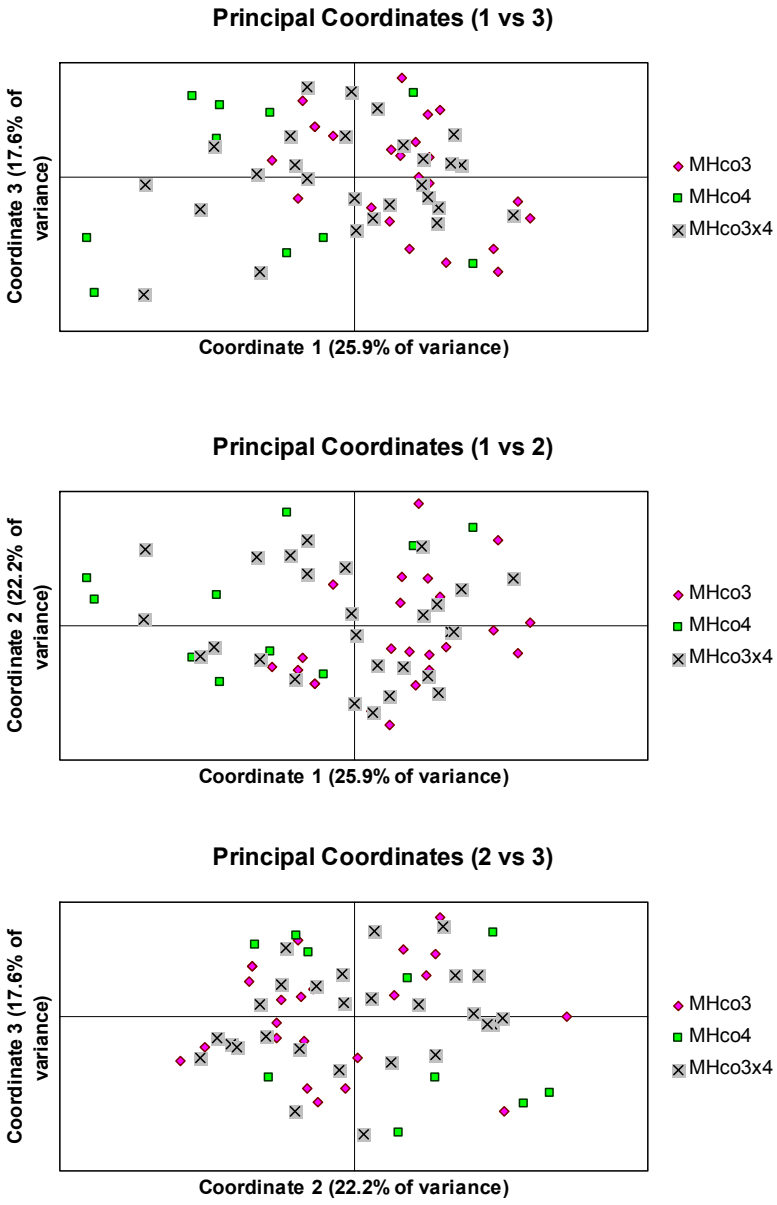


Fig 3.20: Principal coordinates analysis plots for MHco3 (ISE), MHco4 (WRS) and the mixed population of MHco3 (ISE) and MHco4 (WRS) *H. contortus* using a panel of 6 microsatellite loci.

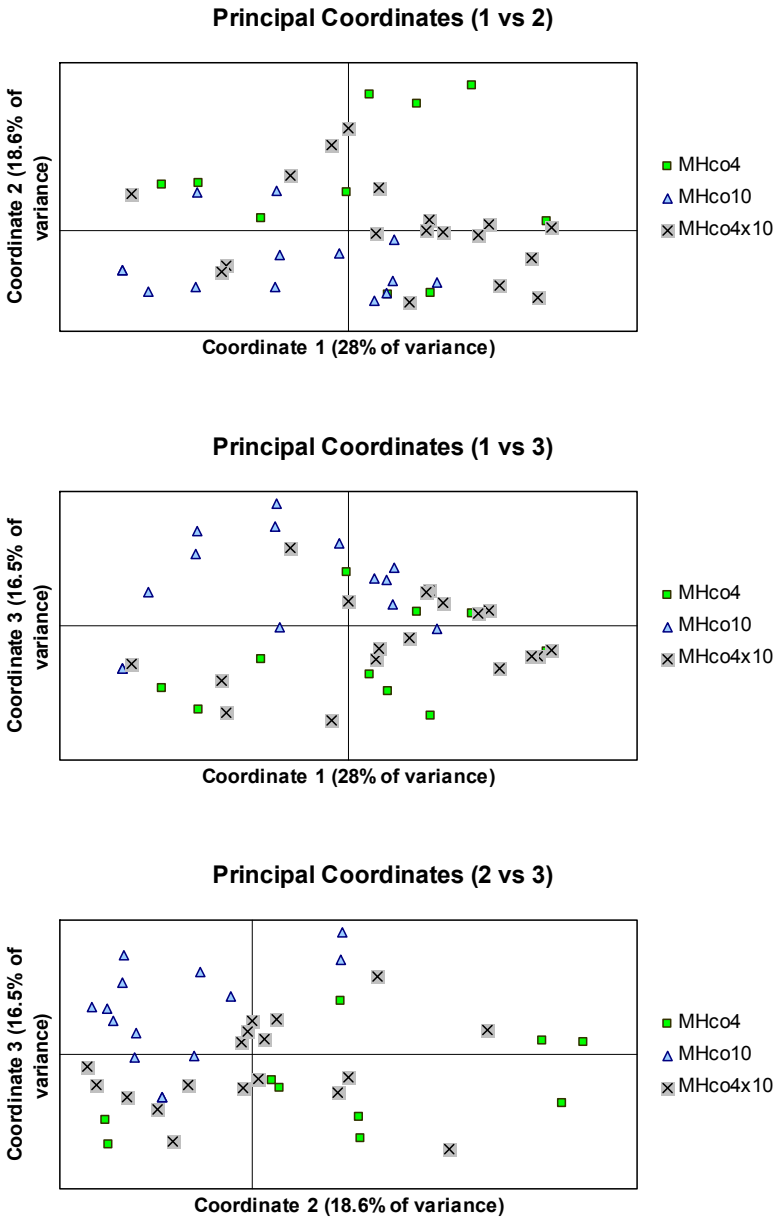


Fig 3.21: Principal coordinates analysis plots for MHco4 (WRS), MHco10 (CAVR) and the mixed population of MHco4 (WRS) and MHco10 (CAVR) *H. contortus* using a panel of 6 microsatellite loci.

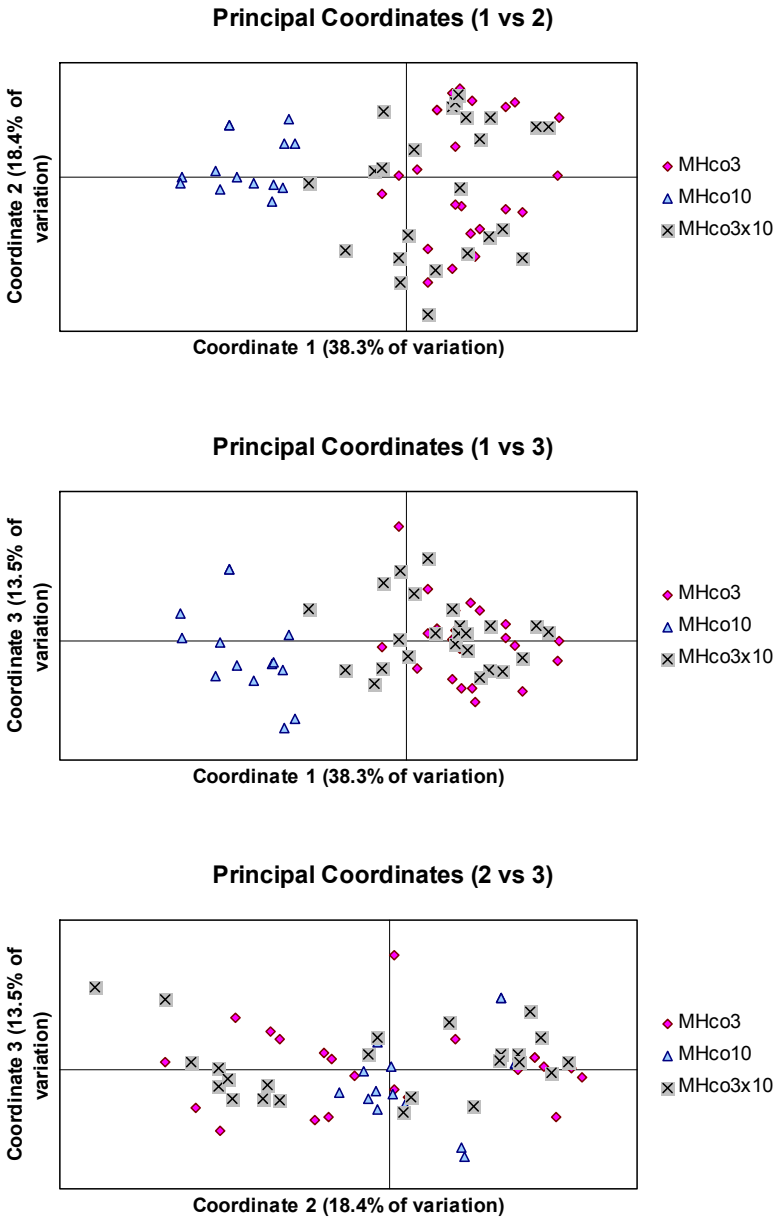


Fig 3.22: Principal coordinates analysis plots for MHco3 (ISE), MHco10 (CAVR) and the mixed population of MHco3 (ISE) and MHco10 (CAVR) *H. contortus* using a panel of 6 microsatellite loci.

3.3.2.4 Effects of environmental conditions on molecular marker allele frequencies of non-parasitic stages of H. contortus

Bulk genescan traces were produced for a panel of 5 microsatellite markers using bulk DNA lysates of L_3 that had been cultured and stored under different conditions, to demonstrate awareness of the fact that L_3 survival could be influenced by genetic factors determining fitness and longevity.

The bulk Genescan traces at five microsatellite loci for lysates of live L_3 cultured and stored under different conditions were more-or-less indistinguishable from each other (for example Fig 3.23 and appendix 3.3), indicating that these loci were not linked to genes conferring survival under the specific conditions.

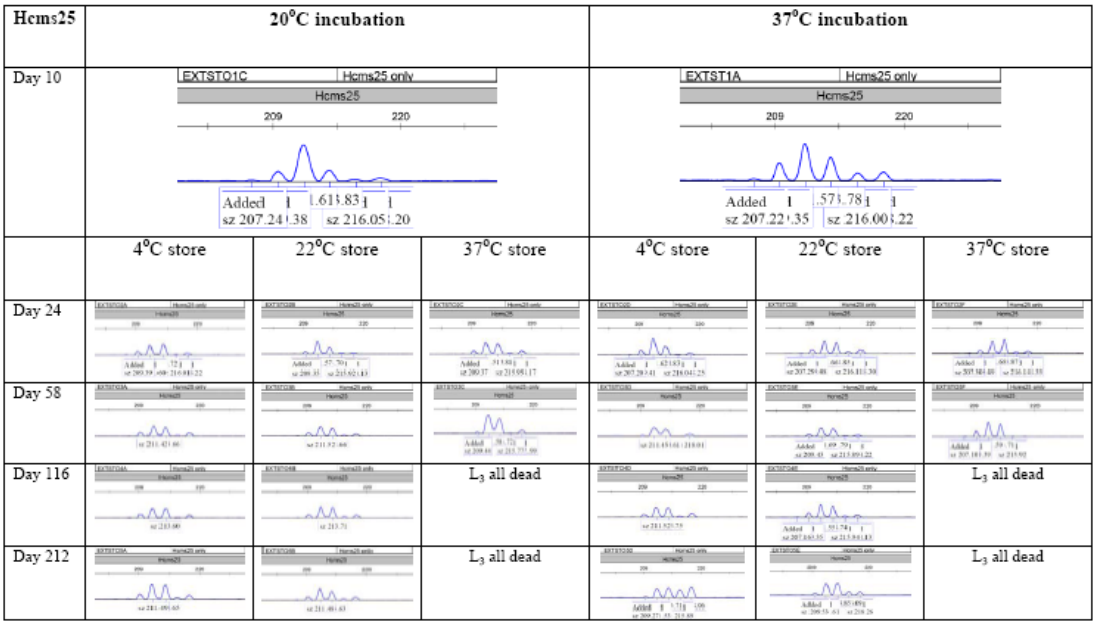


Fig 3.23: Bulk Hcms25 Genescan traces for MHco3 (ISE) *H. contortus* cultured and stored under different conditions. (Clearer traces are shown in appendix 3.3.)

3.3.3 Phenotypic characterisation of isolates and strains of *H. contortus*

3.3.3.1 The larval feeding inhibition assay

The LFIA was an important tool for the *in vitro* characterisation of the ivermectin resistance phenotype of the different isolates and strains of *H. contortus* used throughout this study. However, the assay failed to yield consistent results, when repeated throughout the course of the year using the same characterised isolates. Observational studies were therefore attempted to identify factors that might have led to seasonal alterations in larval feeding in the LFIA. The information presented in this results section (4.3.3.1) is intended to be no more than a series of preliminary observations and/or impressions to highlight the limitations of the LFIA and that might form the basis for discussion and future experimental design.

The LFIAs enabled the production of dose response curves, which could be used to compare different nematode strains (for example, Fig 3.24). Comparisons were confused by poor levels of feeding in drug free controls (for example, MHco10 in Fig 3.24) and at different drug concentrations. Consequently, drug concentrations at which 50% of the L_1 fed (LFI_{50} values) could not be determined. However, discriminatory doses, represented by LFI_{90} and LFI_{99} values enabled comparison of the *H. contortus* isolates, and were broadly correlated with the known *in-vivo* resistance phenotypes.

Valid results, whereby LFI_{90} values could be calculated, were obtained from replicated LFIAs using 15 MHco3 (ISE), 11 MHco4 (WRS) and 10 MHco10 (CAVR) *H. contortus* cultures. The difference between the mean discriminatory doses for the ivermectin susceptible MHco3 (ISE) *H. contortus* strain and the ivermectin resistant MHco4 (WRS) *H. contortus* strain was less than 2 fold and the difference between MHco4 (WRS) and MHco10 (CAVR) was about 2 fold (Fig 3.25). Furthermore the significance of differences between strains was limited by a high level of within strain variation, that

was shown to be partly be related to effects of different donors and times of year, but could also have been because the repeatability of the assay itself was poor.

H. contortus strain	LFI_{50} (µg/ml)	LFI_{90} (µg/ml)	LFI_{99} (µg/ml)
MHco3 (ISE) IVM susceptible *in vivo*	0	0.022	0.035
MHco4 (WRS) IVM resistant *in vivo*	0.029	0.073	0.118
MHco10 (CAVR) IVM resistant *in vivo*	0	0.042	0.089

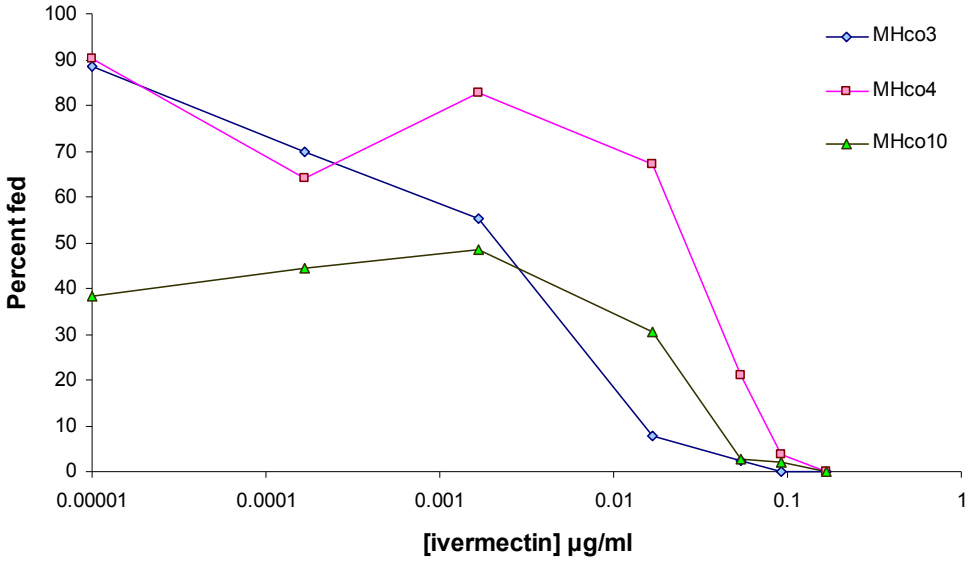


Fig 3.24: LFIA dose response curves and LFI_{50}, LFI_{90} and LFI_{99} values for MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus*. (The assays were set up concurrently on 2nd October using the same ivermectin drug concentrations.)

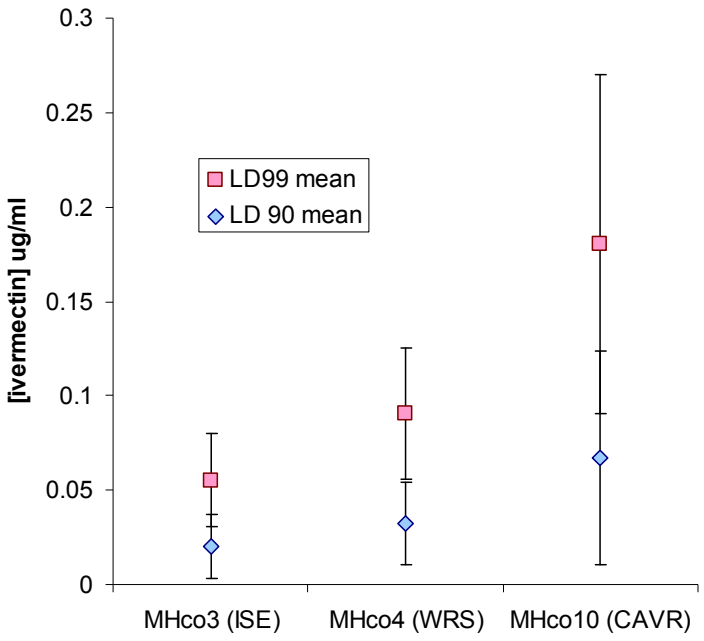


Fig 3.25: Mean (±SD) LDI_{90} and LDI_{99} values for *H. contortus* L_1 harvested from 4 x MHco3 (ivermectin susceptible) donor lambs (15 valid assays), 2 x MHco4 (ivermectin resistant) donor lambs (11 valid assays) and 2 x MHco10 (ivermectin resistant) donor lambs (10 valid assays) conducted over a period of about one year. (Note the high level of within strain variation.)

Storage of faeces at 4°C for 24 hours before egg hatching reduced the rate of egg hatching, but had no subsequent effect on L_1 feeding in the LFIA. The temperature of tapwater used during egg extraction had no noticeable effect on the rate or success of egg hatching or larval feeding. The rate of egg hatching was higher during incubation at 30°C than during incubation at 24°C or 20°C, but while MHco4 (WRS) *H. contortus* L_1 generally fed better at 30°C than at 24°C, MHco3 (ISE) L_1 did not feed at 30°, probably due to exhaustion of energy reserves following higher metabolic rates when incubated at the higher temperature. Feeding of both MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* was generally poor following hatching and incubation at 20°C, probably associated with an increased opportunity for infection with pathogenic fungi or bacteria during the prolonged egg hatching period. Oxygenation of the water used for egg hatching and larval incubation had no observable effect on larval feeding. However, the

source of water did have an apparent effect, with consistently better feeding in tapwater than in distilled water.

The effect of the pH of the water used for egg hatching and larval incubation was determined using NaH_2PO_4 buffered distilled water at different pH levels. While egg hatching was generally optimal between pH 7.0 and 7.4, there were apparent differences in larval feeding between the two strains, with optimal feeding in the MHco3 (ISE) and MHco4 (WRS) strains in slightly acid and alkaline conditions respectively. However, the percentage of larvae feeding was low in all cases and evidence of fungal, or perhaps bacterial, contamination was seen in several samples, indicating that the results were confounded by differences in conditions other than pH.

The relevance of fungal infection was investigated by washing eggs for 5 minutes in 0.15% sodium hypochlorite solution before incubation, and comparing egg hatching and larval feeding following incubation in different anti-fungal drugs. Washing eggs in sodium hypochlorite solution improved larval feeding in some cases, although the effect was inconsistent. Inclusion of 50μg/ml amphotericin (final concentration) to the water used for egg hatching and larval incubation generally improved larval feeding, although the poor solubility of the drug caused impracticalities when examining the larvae and >90% feeding was only achieved on a few occasions. (Data to support these statements are available, but are not presented here because they are insubstantial relative to the overall content of this thesis.)

It is possible that the extensive and generalised fungal infection that was seen in some L_1 (for example, Fig 3.22 C) originated in the trichostrongyle eggs within their sheep host, rather than in the water used for egg hatching and larval incubation. Thus, the poor feeding in the LFIA could be related to various different sources of fungal or bacterial contamination.

A MHco4 (WRS) *H. contortus* donor lamb was euthanased in February 2006, providing an opportunity to recover adult *H. contortus*. (Problems of fungal contamination in the LFIA had consistently been observed affecting L_1 hatched from eggs voided in the faeces of this donor.) Adult *H. contortus*, along with eggs and developing larvae, cultured from both sodium hypochlorite-washed and unwashed eggs, collected from the same donor host were incubated on Sabourad's agar for 14 days. No fungi were cultured from the adult *H. contortus*. Only white fungal colonies were cultured from the eggs, and both green and white fungal colonies were cultured from the L_1 and L_3 (Fig 3.26). Each of the white colonies and each of the green colonies had the same distinct morphological characteristics, but fungal species identification was not possible. The same results were achieved from eggs and developing larvae, irrespective of whether or not the eggs had been washed in sodium hypochlorite. These observations would support the hypothesis that the LFIA results were confounded by two separate fungal contaminants, one originating within the donor sheep (white colonies) and one originating from the water used for egg hatching and larval incubation.

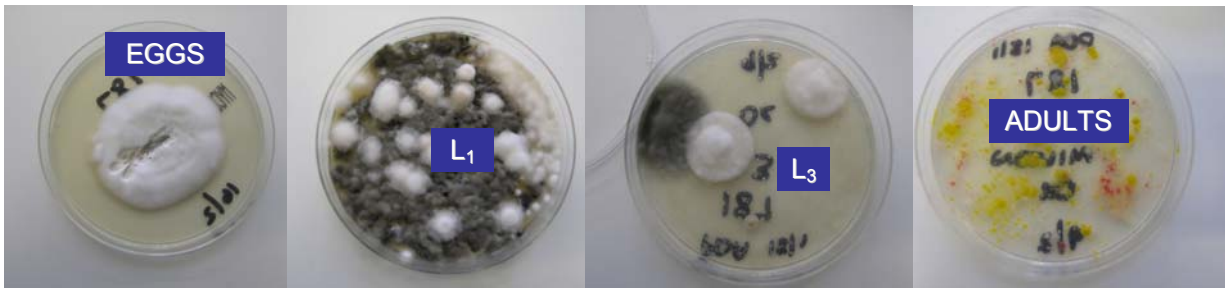


Fig 3.26: Fungal colonies on Sabourad's agar, inoculated with MHco4 *H. contortus* eggs (white colonies), L_1 (green and white colonies), L_3 (green and white colonies) and adults (no fungal growth).

3.3.3.2 The effects of donor age and immunity

The previous section illustrates just some of the problems that arose throughout the studies presented in this thesis associated with the use of older donor or recipient sheep. These problems were not necessarily solely due to the age of the donor sheep, and may have been confounded by extrinsic seasonal factors. Aspects of *H. contortus*

parasitology were therefore compared between older 14 month-old worm-free donor sheep (hoggs) and young 3 month-old lambs infected once with the same sized dose of L_3 of the three different laboratory strains of *H. contortus* (one lamb and four hoggs were infected with each strain). The effects of corticosteroid immune suppression were investigated in the hoggs.

The FWECs of the three lambs rose sharply between days 17 and 27 to 32, before dropping to about half of their maximal levels, while the FWECs of the hoggs only rose slowly from day 20 to about half the day 45 levels of the lambs infected with the same *H. contortus* strains. The FWECs of lambs and hoggs infected with MHco10 (CAVR) *H. contortus* were consistently lower than those of lambs and hoggs infected with MHco3 (ISE) or MHco4 (WRS) *H. contortus*. Weekly methyl progesterone acetate (MPA) treatment of 14 month-old hoggs had no discernable effect on their FWECs when compared to the untreated hoggs. The FWECs of lambs and hoggs infected with MHco3 (ISE), MHco4 (WRS), or MHco10 (CAVR) *H. contortus* are shown in Fig 3.27.

The mean number of adult MHco3 (ISE), MHco4 (WRS) or MHco10 (CAVR) *H. contortus* recovered from the abomasa of lambs, hoggs or hoggs treated with MPA was between 1800 and 3000, with no clear difference between any group or strain of *H. contortus* (Fig 3.28).

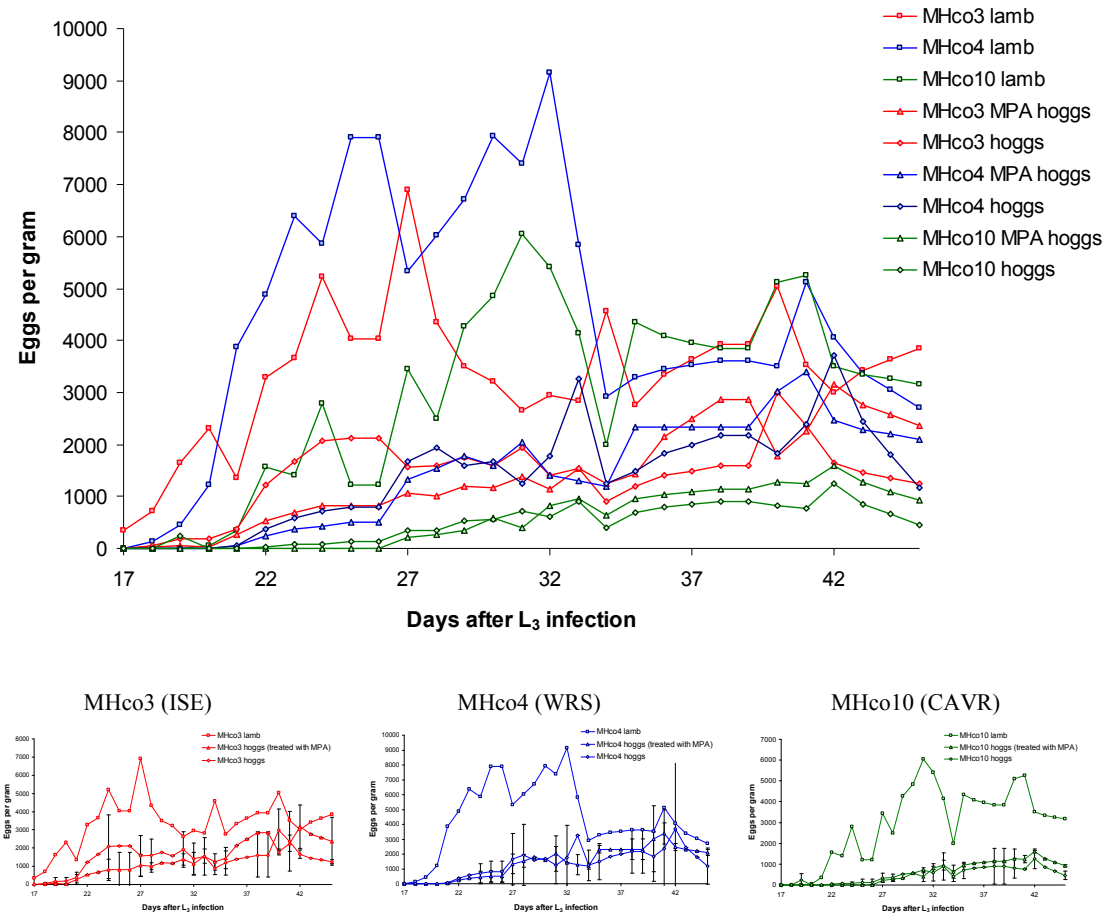


Fig 3.27: FWECs of 3 - 4 month-old lambs, 14 – 15 month-old hoggs (mean [±SD] values) and 14 – 15 month-old hoggs treated with MPA (mean [±SD] values), following infection with 5,000 MHco3 (ISE), MHco4 (WRS), or MHco10 (CAVR) *H. contortus* L_3 on day 1.

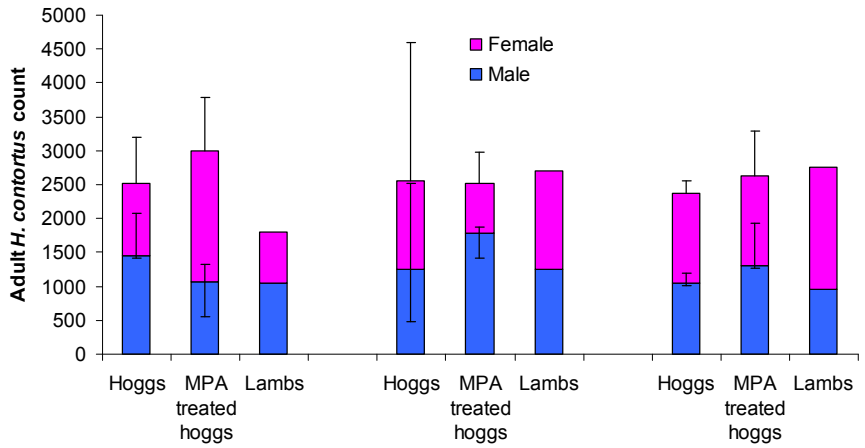


Fig 3.28: Total postmortem day 45 adult *H. contortus* counts of three groups of sheep infected with three strains of *H. contortus*.

The hoggs produced on average 836 g of faeces over a 24 hour period between days 44 and 45, while the lambs (which were fed on about twice the amount of concentrate feed and more palatable hay than the hoggs) produced on average 1.6 kg of faeces over the same period. Comparison of the day 45 FWECs and weight of faeces produced per day with the number of adult female *H. contortus* identified in the abomasum on day 45 enabled an estimate to be made of the number of eggs shed per adult female *H. contortus* per day. Adult female *H. contortus* in the abomasa of 4 month-old lambs shed 6.5, 3.1 and 8.0 times more eggs than adult female *H. contortus* in the abomasa of 15 month-old hoggs (mean of 4 hoggs/group) infected with MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* respectively. Adult female *H. contortus* in the abomasa of 15 month-old hoggs treated weekly with MPA shed 1.5, 2.1 and 1.9 times more eggs than adult female *H. contortus* in the abomasa of the untreated 15 month-old hoggs infected with MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* respectively (Fig 3.29). *H. contortus* within the abomasum of the 4 month-old lamb infected with MHco3 (ISE) *H. contortus* shed an average of 3500 eggs per day.

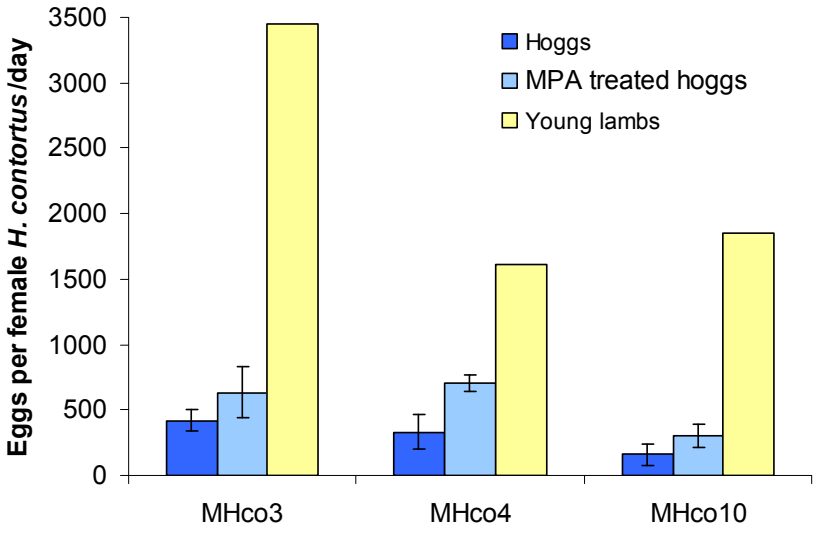


Fig 3.29: Estimated day 45 egg shedding by adult female MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* in the abomasa of 4 month-old lambs, 15 month-old hoggs and 15 month-old hoggs treated with methyl progesterone acetate.

The L_3 yield of the coprocultures was high in every case, and significant differences between groups were not identified. Examples are shown in Fig 3.30. (It was commonplace throughout the course of study for the number of L_3 recovered to exceed the number of eggs estimated have been incubated, based on FWECs. This reflects the fact that the efficiency of coproculture is close to 100%, while saturated salt floatation methods of counting trichostrongyle eggs in faeces probably underestimate their numbers. Furthermore, the FWEC of the subsample of faeces that was counted may not have equalled that of the subsample that was cultured.)

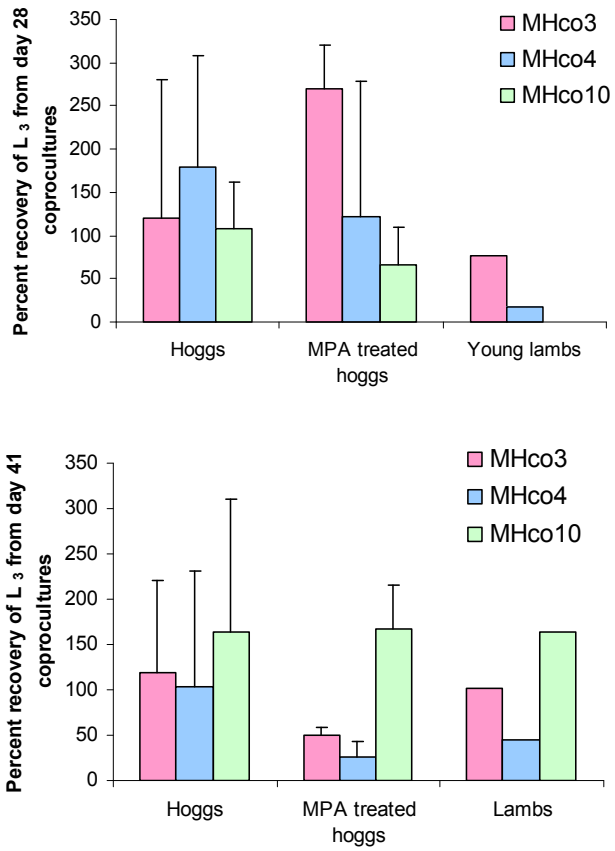


Fig 3.30: Efficiency of L_3 recovery from coprocultures.

The ED_{50} values calculated in the EHAs (Fig 3.31) were consistent with those for the benzimidazole susceptible *in vivo* MHco3 (ISE) and MHco10 (CAVR) *H. contortus*, and benzimidazole resistant *in vivo* MHco4 (WRS) *H. contortus* elsewhere in this study. No significant difference in ED_{50} values was identified within groups between 4 month-old lambs, 15 month-old hoggs and 15 month-old hoggs treated weekly with MPA.

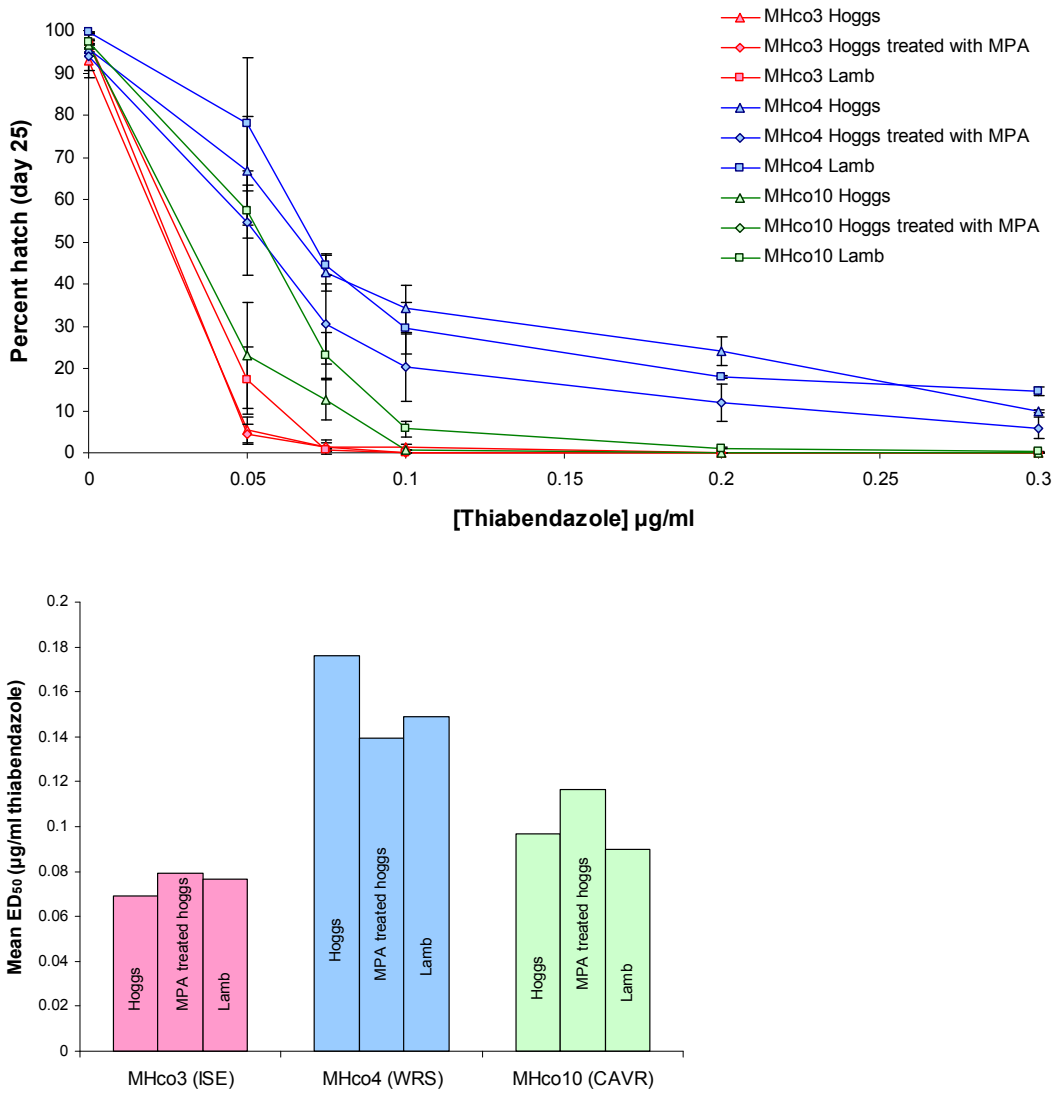


Fig 3.31: Dose response curves for EHAs using MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* infections of 4 - 5 month-old lambs, 14 – 15 month-old hoggs and 14 – 15 month-old hoggs treated with MPA. ED_{50} values for MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus*.

The LFIAs produced inconsistent results, partly due to erratic feeding in drug free controls and in low drug concentrations (for example, Fig 3.32). The LFI_{90} and LFI_{99} values for the MHco4 (WRS) strain of H. contortus were consistently higher than those for the MHco3 (ISE) strain of *H. contortus*, reflecting their ivermectin resistance status *in vivo*. However, the LFIA failed to discriminate between the ivermectin susceptible *in*

vivo MHco3 (ISE) and ivermectin resistant *in vivo* MHco10 (CAVR) strains of *H. contortus* (Fig 3.33). The results were confounded by poor levels of feeding in the drug free controls, affecting the MHco10 (CAVR) strain of *H. contortus* in particular (Fig 3.33). No significant differences were seen in the dose response curves and discriminatory drug concentrations between the hoggs, the hoggs that were treated with MPA and the lambs infected with any of the three reference strains of *H. contortus*.

No significant change was seen in the discriminatory drug concentrations between 27 and 40 days after infection of the sheep with *H. contortus* L_3 (Fig 3.34).

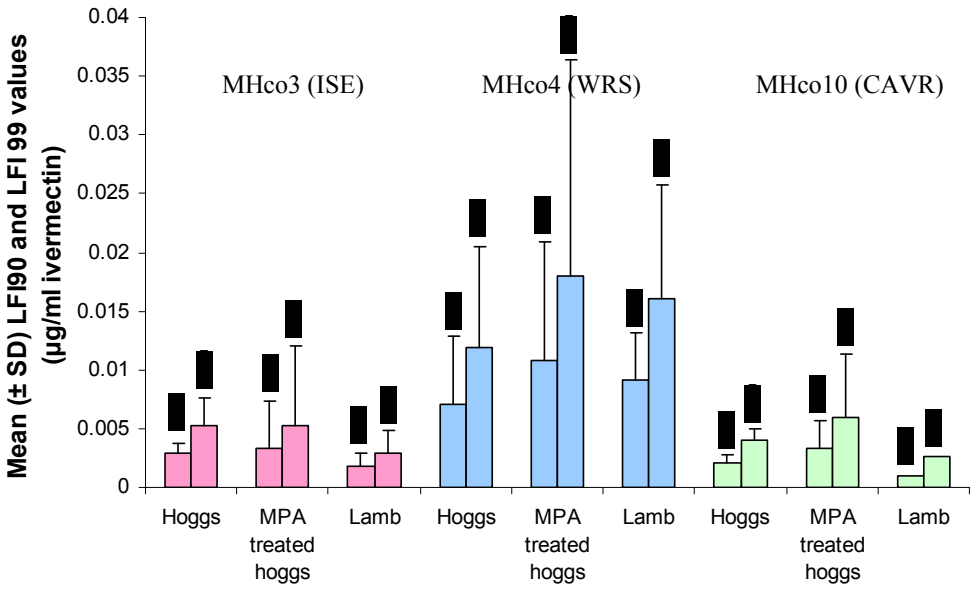


Fig 3.32: i) Mean (+ standard deviations) discriminatory drug concentrations calculated by Probit analysis for different groups of lambs and hoggs.

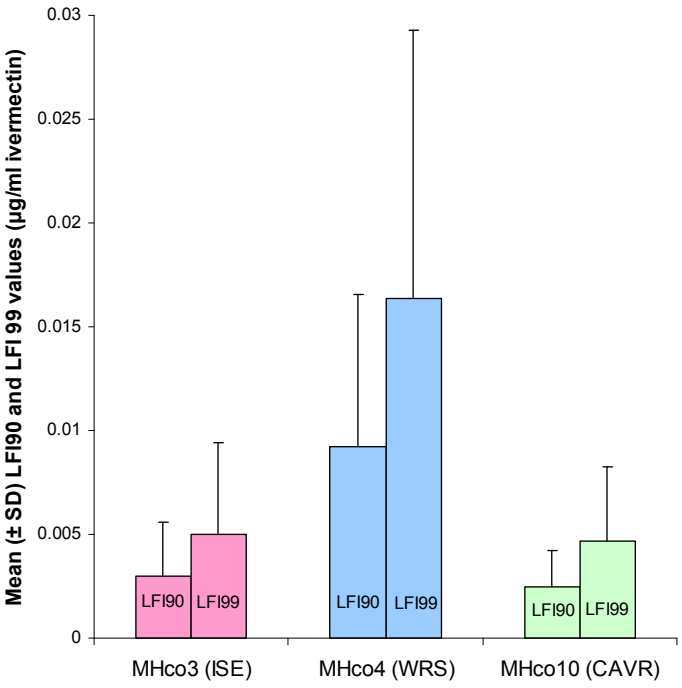


Fig 3.32: ii) Average LD_{90} and LD_{99} values of sheep infected with MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus*.

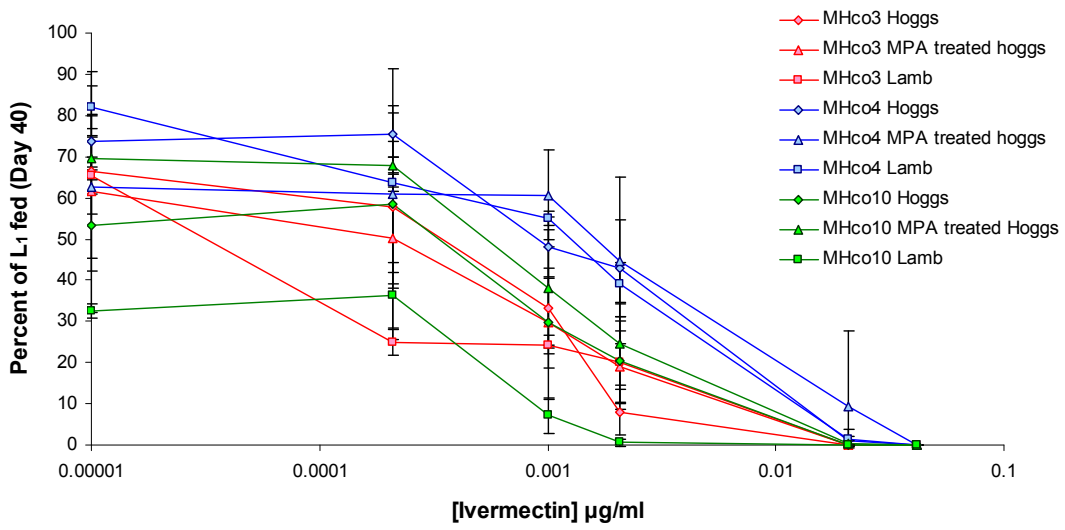


Fig 3.33: An example of dose response curves produced in the LFIA, showing erratic feeding in drug free controls, affecting the MHco10 (CAVR) strain of *H. contortus* in particular.

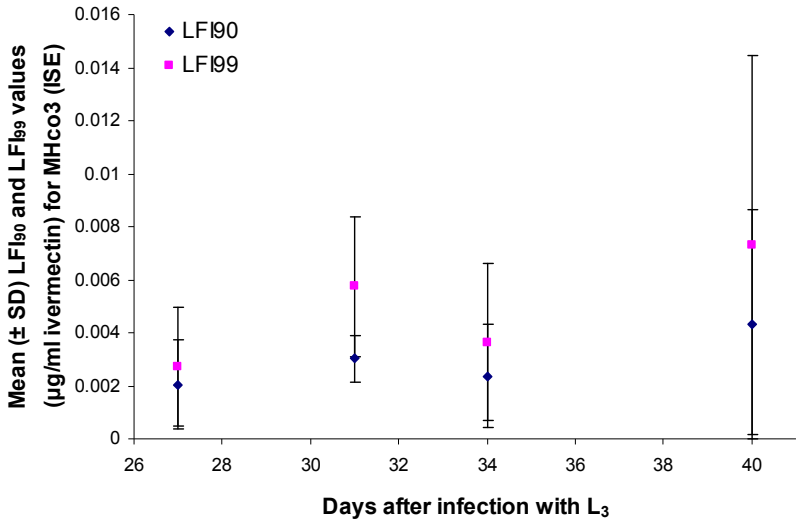


Fig 3.34: Average (± standard deviations) LFI_{90} and LFI_{99} values at different times following infection of hoggs, hoggs treated with MPA and lambs with MHco3 (ISE) *H. contortus*.

3.3.3.3 Investigation of the level of dominance/recessivity of the resistance trait

Twenty male MHco3 (ISE) (benzimidazole and ivermectin susceptible) and 20 female MHco4 (WRS) (benzimidazole and ivermectin resistant) day 14 *H. contortus* late L_4/immature adult *H. contortus* were surgically transferred into the abomasum of a worm-free recipient lamb. Two worm-free lambs (donors A and B) were infected with about 5,000 F_1 L_3 recovered from the coprocultures of the recipient lamb and used to produce F_2 nematodes. Support of a successful genetic cross was sought using microsatellite DNA fingerprinting of the F_1 and F_2 nematodes and determination of dominance or recessivity of the anthelmintic resistance trait in the MHco4 (WRS) strain of *H. contortus* was based on measurement of the phenotypic expression of resistance in the F_1 and F_2 nematode populations.

The FWECs of the recipient lamb are shown in Fig 3.35. Fifteen female *H. contortus* and 2 male *H. contortus* were recovered from the abomasum of the recipient lamb, 184

days after the surgical transfer of 20 male and 20 female, day 14 late L_4/immature adult *H. contortus*.

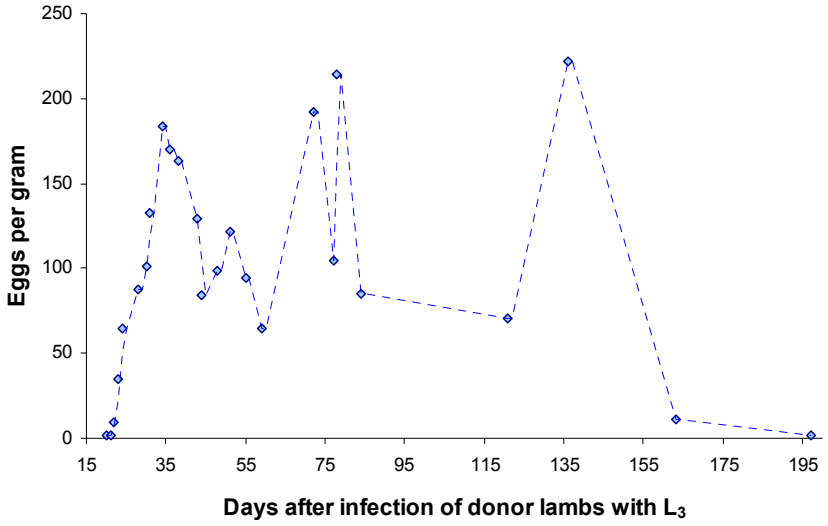


Fig 3.35: F_1 FWECs of the recipient lamb following surgical transfer of 20 male MHco3 and 20 male MHco4 *H. contortus* on day 14.

The allele frequencies of 37 F_1 L_3 at microsatellite loci Hcms15, Hcms27, Hcms94 and Hcms22co3 are shown in Fig 3.36 along with reference data (Libby Redman, *data on file*) for the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus*. The predicted allele frequencies for a genetic cross between 100 MHco3 (ISE) and 100 MHco4 (WRS) *H. contortus* parents (ignoring the contribution of null alleles) were crudely estimated from for the sum of the reference MHco3 (ISE) and MHco4 (WRS) *H. contortus* allele frequencies divided by 2. The F_1 allele frequencies approximate, but are not wholly consistent with those predicted on the basis of a successful mass mating genetic cross having been successfully undertaken. For example, allelic dropout in the F_1 progeny of the genetic cross at locus Hcms94 is apparently inconsistent and requires explanation. The microsatellite genotypes of the individual L_3 F_1 progeny of the genetic cross between 20 male MHco3 (ISE) and 20 female MHco4 (WRS) *H. contortus* are shown in appendix 3.4.

The FWECs of the two F_2 donor lambs (A and B) are shown in Fig 3.37. The day 17 post full and half dose ivermectin treatment efficacies for F_2 donor lambs A and B were 98% and 0 respectively. Correspondingly, 20 and about 1000 adult *H. contortus* were recovered from the abomasa of lambs A and B respectively at postmortem 77 days after treatment on day 37 with 200 μg/kg and 100 μg/kg ivermectin respectively.

Microsatellite locus	Allele	F_1 progeny of the 20 MHco3 x 20 MHco4 genetic cross	MHco3 *H. contortus*	MHco4 *H. contortus*
Hcms15	272	48.7%	45%	0
	287	51.3%	55%	100%
Hcms27	338	3.9%	3.7%	41.3%
	344	17.3%	0	21.7%
	348	0	0	4.4%
	358	78.8%	96.3%	32.6%
Hcms94	172	0	0	11.5%
	174	0	0	13.5%
	177	0	0	3.8%
	193	0	3.3%	1.9%
	194	0	0	1.9%
	196	0	0	15.4%
	201	0	0	3.9%
	225	0	1.7%	0
	231	0	0	3.9
	233	100%	95%	26.9%
	235	0	0	13.5%
Hcms22co3	234	15%	7.3%	29.8%
	242	2.5%	0	34%
	250	70%	92.7%	19.9%
	258	12.5%	0	16.2%

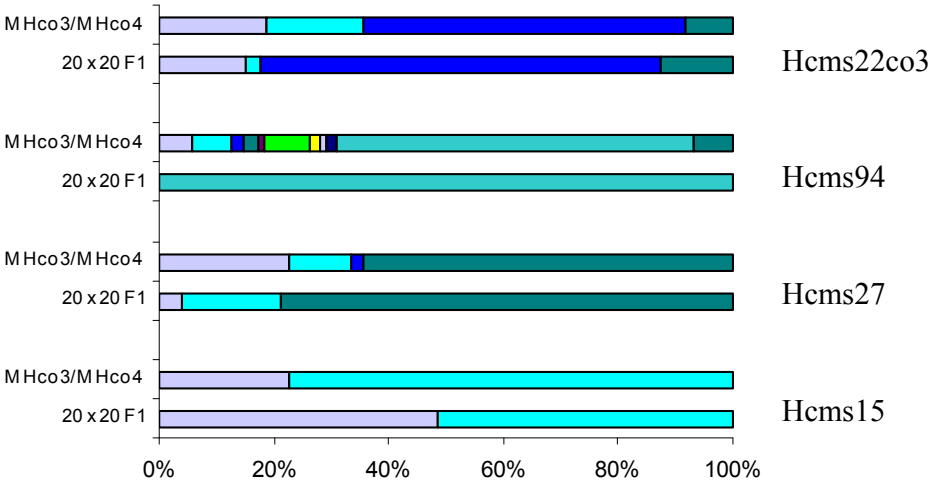


Fig 3.36: Schematic representation of the allele frequencies at 4 microsatellite loci for the F_1 progeny of a genetic cross between 20 male MHco3 and 20 female MHco4 *H. contortus* (20 x 20 F_1). The expected allele frequencies of a genetic cross between 100 MHco3 and 100 MHco4 *H. contortus* parents (MHco3/MHco4) are compared with the allele frequencies for the F_1 progeny of the genetic cross between 20 male MHco3 and 20 female MHco4 *H. contortus*. Each colour in the bar represents the frequency of a particular allele and the same colours are used for the same alleles for each marker.

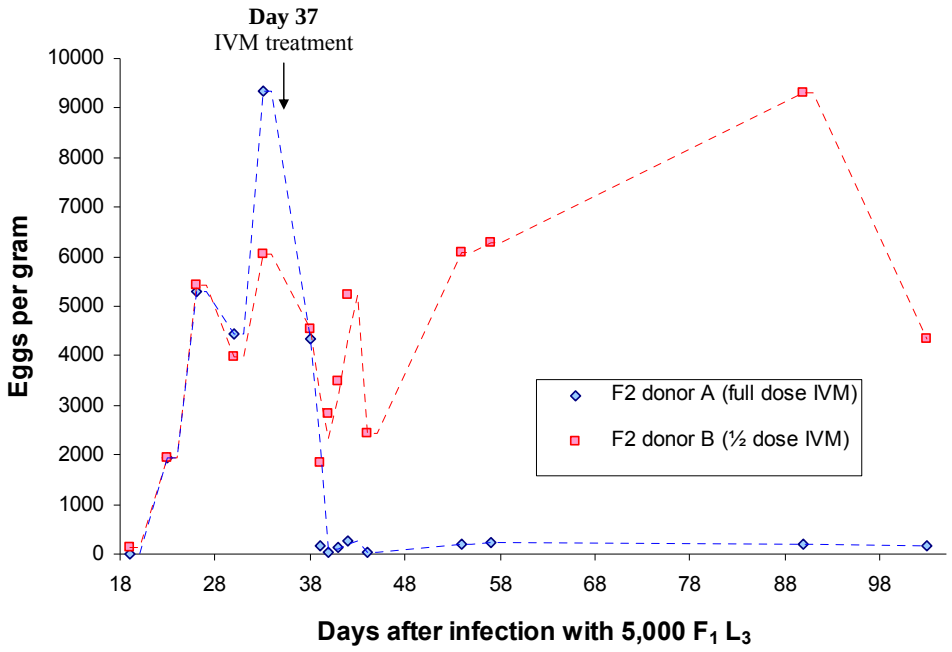


Fig 3.37: FWECs of two donor lambs infected with 5,000 F_1 L_3 on day 1 and treated with ivermectin on day 37.

LFI_{90} and LFI_{99} values obtained from the LFIAs using F_1 and F_2 progeny of the 20 male (MHco3) and 20 female (MHco4) parent genetic cross are compared to values obtained from contemporary donor sheep infected with MHco3 and MHco4 strains of *H. contortus* (Table 3.7). The resistance phenotype of the F_1 population in the LFIA was similar to that of the resistant MHco4 *H. contortus* parent strain, while the level of resistance (based on LFI_{99} values) was higher in the F_2 populations. These results are consistent with phenotypic traits measured using the LFIA being dominant (F_1: RS [resistant]. F_2: RR [resistant]: 2RS [resistant]: SS [susceptible]). Overall, no difference in resistance phenotype was detected in F_2 *H. contortus* populations before treatment with a full or half therapeutic oral dose of ivermectin and F_2 *H. contortus* populations surviving ivermectin treatment. The standard deviations in $LFIA_{90}$ and $LFIA_{99}$ values obtained for the different F_1 and F_2 cohorts were high, and these differences and similarities when tested by Chi-square are not statistically significant. The dose response curves for LFIAs and EHAs for F_2 populations of L_1 and eggs are shown in Fig 3.38.

H. contortus strain	LFIA LFI_{90} (µg/ml IVM) [±SD]	LFIA LFI_{99} (µg/ml IVM) [±SD]	EHA ED_{50} (µg/ml TBZ) [±SD]
MHco3 (ISE)	0.028 [±0.011] (4)	0.044 [±0.014] (4)	0.048 [±0.017] (3)
MHco4 (WRS)	0.057 [±0.013] (4)	0.075 [±0.049] (4)	0.236 [±0.1] (2)
MHco3/MHco4 F_1	0.041	0.062	0.054
MHco3/MHco4 F_2 A (pre IVM treatment	0.049 [±0.027] (2)	0.086 [±0.045] (2)	No data
MHco3/MHco4 F_2 B (pre ½IVM treatment	0.044 [±0.034] (2)	0.077 [±0.058] (2)	0.2
MHco3/MHco4 F_2 A (post IVM treatment	0.044 [±0.001] (2)	0.096 [±0.023] (2)	0.112 [±0.025] (2)
MHco3/MHco4 F_2 B (post ½IVM treatment	0.065 [±0.034] (2)	0.118 [±0.057] (3)	0.132 [±0.033] (3)

Table 3.7: ED_{50} values obtained from egg hatch assays and LFI_{90} and LFI_{99} values obtained from larval feeding inhibition assays using F_1 and F_2 progeny of the 20 male (MHco3) and 20 female (MHco4) parent genetic cross and eggs or L_1 from contemporary donor sheep infected with MHco3 and MHco4 strains of *H. contortus*. Standard deviations are calculated using the results of different numbers of assays that were reliably performed (shown in brackets).

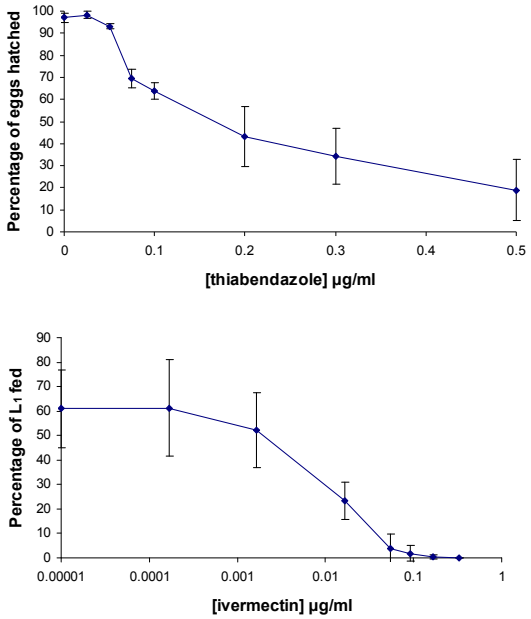


Fig 3.38: Dose response curves produced for bioassays using the F_2 population. Error bars show standard deviations between replicates.

The benzimidazole resistance phenotype of the F_1 population in the EHA was susceptible, while that of the F_2 populations was resistant and intermediate between the MHco3 (ISE) and MHco4 (WRS) parent populations (Table 3.7). Based on simple Mendelian principles, these results are consistent with the benzimidazole resistant phenotype in the EHA being recessive (F_1: RS [susceptible]. F_2: RR [resistant]: 2RS [susceptible]: SS [susceptible]).

The adult *H. contortus* of the parental strains that were recovered from the abomasum of the recipient sheep on autopsy were genotyped for the isotype 1 β tubulin F200Y polymorphism. The genotypes of 6 female parent (MHco4) *H. contortus* were *TT* (homozygous benzimidazole susceptible) and 7 were *AT* (heterozygous). Two male parent (MHco3) *H. contortus* were *TT* and *AT* (the P200Y SNP allele frequencies of MHco3 *H. contortus* determined throughout this study are 0.95*T*: 0.05*A*). One female *H. contortus* that was *AT* at F200Y was also *AT* at F167Y, while the other *H. contortus* were all *TT* at F167Y. Thirty-one MHco3 (ISE) eggs that did not hatch in a concentration of 0.1μg/ml of thiabendazole in the EHA were all *TT* (homozygous susceptible) at F200Y, while 7 and 18 MHco3 *H. contortus* L_1 that hatched in a concentration of 0.1μg/ml of thiabendazole in the EHA were *AT* and *TT*, respectively, at F200Y. All of the MHco3 *H. contortus* eggs and L_1 recovered from the EHA were *TT* at F167Y. These results are summarised in Fig 3.39 showing that neither parent population was genotypically monomorphic, and that the egg hatching to L_1 phenotype in the EHA is not solely determined by the F200Y genotype.

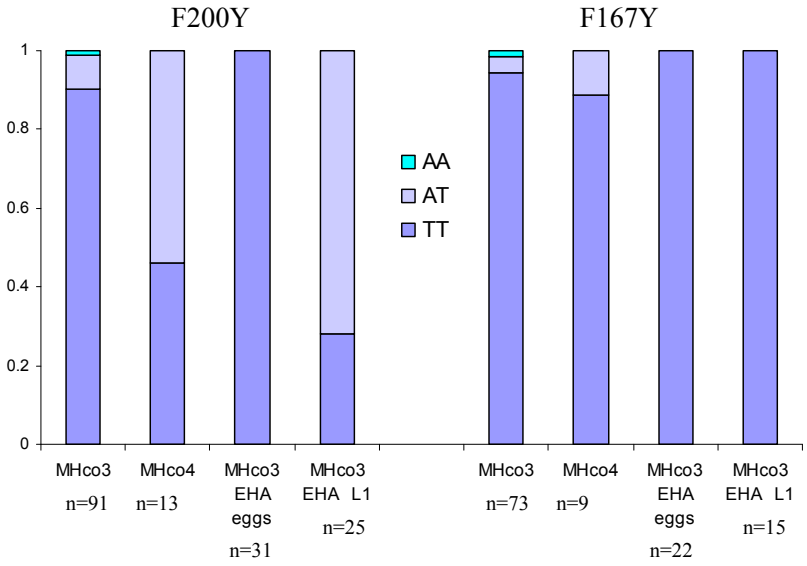


Fig 3.39: Isotype 1 β tubulin F200Y and F167Y genotype frequencies for MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for MHco3 *H. contortus* benzimidazole susceptible eggs and resistant L_1 recovered from a concentration of 0.1 µg/ml thiabendazole in the EHA. (n: number of individual nematodes genotyped.)

3.4 Discussion

3.4.1 Development of a method to enable successful recovery of the progeny of single *H. contortus* parents

3.4.1.1 Success of single parent genetic crosses

Conclusive evidence could not be found to suggest that the transfer of single male MHco3 and single female MHco4 late L_4/immature adult *H. contortus* to the abomasa of recipient sheep resulted in a successful genetic cross. Positive FWECs of recipient lambs might have been consistent with the production of between 100 and 1000 eggs per day by a single female *H. contortus*, but in most cases these counts were observed several days later than would have been expected following the maturation of the single

day 14 late L_4/immature adult *H. contortus* that were surgically transferred. Furthermore, *H. contortus* was only conclusively demonstrated in the coprocultures of 2 of the 6 recipients. While it is possible that a successful single parent genetic cross occurred, but was not confirmed due to subsequent contamination or (less likely) due to shortcomings of the markers used; the failure to provide conclusive proof shows that the probability of single male and female day 14 *H. contortus* surviving to maturity and then finding each other and mating following surgical transfer to the abomasa of recipient sheep is very low. It is nevertheless possible that following a larger number of rounds of surgical transfer, the method might eventually lead to the confirmation of a successful single day 14 *H. contortus* parent genetic cross. While this could perhaps be achieved, aided by technologies such as the use of abomasal fistulated sheep, or protection of the nematodes within gelatine capsules or a permeable membrane (unlikely to be successful for blood feeding *H. contortus*), these preliminary studies show the need to consider alternative genetic crossing methods to enable a genome wide search for candidate genes conferring anthelmintic resistance.

FWEC data collected from donor sheep throughout this study suggest that MHco3 (ISE) and both MHco4 (WRS) and MHco10 (CAVR) *H. contortus* generally reach maturity (based on commencement of fertile egg shedding) from about days 17 and 19 respectively following infection. A previous study (Le Jambre, 1993) showed that other CAVR strains of *H. contortus* may not commence egg shedding until 24 days after L_3 infection. The experimental protocol involving surgical transfer of day 14 late L_4/immature adult *H. contortus* was chosen to ensure that the female nematodes would not have mated before they were transferred, thus guaranteeing that any progeny would be of the single male parent. However, the parasitic nematode mortality rate and probability of the single male and female finding each other and mating would be proportional to time interval between surgical transfer and maturity. The problem caused by a 3 to 5 day interval between surgical transfer of day 14 nematodes and maturity could be overcome by the transfer of single mature adult *H. contortus*, but in the absence of information concerning how long female nematodes continue to shed

fertilised eggs following mating, the contribution of the single male parent to the progeny would be unknown. In populations of *H. contortus*, mating has been shown to be polyandrous, with single females shedding eggs fertilised by up to at least 4 males (Redman and others, *In Press*). Determination of the period of time that female *H. contortus* continue to shed fertilised eggs following the removal of males would therefore be useful. If this is shown to be longer than 3 days, during which one of the single nematodes could be lost from the abomasum, then transfer of adult *H. contortus* would not be helpful and alternative methods to ensure single parent mating should be sought.

3.4.1.2 Parasitic nematode contamination

The preliminary attempts to achieve a successful single parent genetic cross demonstrated problems of parasitic nematode contamination of the coprocultures. Four of the six recipient sheep showed contamination with different parasitic nematode species (*T. circumcincta*, *Trichostrongylus* spp. and/or *N. helvetianus*, as well as with free living or partially parasitic *Strongyloides* spp.). Another sheep produced only eggs of *H. contortus* but molecular genetic analysis showed this not to be the result of a single pair mating, but the result of contamination by *H. contortus* from another source. This contamination could have occurred associated with the rearing, housing and feeding management of the recipient lambs, or in the coproculture room. The lambs used in the study are reared indoors on straw bedding with their anthelmintic treated dams under conditions that should minimise the risk of accidental nematode infection. Subsequently recipient lambs were housed in individual pens and in a different building from non-*H. contortus* donor sheep. L_3 would not be expected to survive on urine- soiled bedding (Helle and others, 1989), or in the dry hay that was fed, although it is possible that these sources could become temporarily contaminated with occasional insect- or fomite-borne larvae. Following the recognition of a contamination problem during the first attempt at a single parent genetic cross (recipients A, B and C), recipient sheep were bedded on unpalatable sawdust rather than straw and the hay was autoclave sterilised (with the benefit of hindsight this procedure was probably unhelpful, and simply made the hay

less palatable). Consideration of the possibility of occasional L_3 contamination of the environment in which the recipient sheep were housed indicated the need to develop a different method that involved recovery of eggs from recipient sheep within a few days of surgical transfer, thus minimising the opportunity for any contaminant L_3 to develop to mature adults.

The major source of contamination was thought to have occurred within the coproculture room. This room regularly became infested with muscid flies. Maggots and juvenile flies were commonly observed in the sheep faeces, and Baermannisation of dead flies, collected from the tray of an electrocution device placed in the culture room, repeatedly yielded both free living and sheathed parasitic nematode larvae. Baermannisation of faeces collected from anthelmintic treated worm-free donor sheep and then placed for 10 days in open trays in the coproculture room consistently yielded large numbers of free living nematode larvae, along with smaller numbers of sheathed parasitic larvae. No *Trichostrongylus* spp. or *N. helvetianus* donor sheep were kept at the Moredun Research Institute concurrently with the second single parent genetic crossing study, so the source of contamination must have been external. Furthermore, *N. helvetianus* eggs require cold priming before they can hatch, so could not have hatched from the coprocultures and must have been imported from an external source. The contaminant *H. contortus* from the second single parent genetic crossing study differed in various respects from the MHco3 (ISE), MHco4 (WRS) or MHco10 (CAVR) strains of *H. contortus* that were maintained at the Moredun Research Institute. For example, it differed from the MHco3 (ISE) and MHco10 (CAVR) strains in being resistant to thiabendazole in the EHA and differed from the MHco3 (ISE) strain in the dose response curves produced in the LFIA, while its population genetic fingerprint using a panel of just four neutral markers was clearly different from that of the MHco4 (WRS) and MHco10 (CAVR) strains. In fact, the contaminant *H. contortus* was similar to the Moredun field isolate (and another local isolate) in the bioassays and in its population genetic fingerprint based on a panel of five microsatellite markers. Together this information indicates that the main source of contamination of the coprocultures resulted

from the passive transfer of L_1, L_2 or L_3 from the outside environment on the legs of female flies which were attracted to the odour of faeces either in the animal pens, or more likely in and around the coproculture room in order to lay their eggs. Fly borne *H. contortus* contamination of the coprocultures of recipient D probably originated from the high level of pasture larval contamination that would have arisen from the concurrent outbreak of haemonchosis in lambs in adjacent fields. Subsequent to the identification of flies as the most likely source of larval contamination, coprocultures involved with single parent mating studies were all performed in a closed 24^{o}C incubator, sited within the main parasitology laboratory.

The identification of a probable role of coprophagous insects as vectors of parasitic nematode larvae raises questions about their effect on nematode parasite population genetics. For example, are coprophagous insects involved in the contamination of clean pasture, in the introduction of new strongylid nematode populations to sheep flocks, or in the transmission of anthelmintic resistance between flocks?

3.4.1.3 Molecular genetic proof of a genetic cross

The preliminary studies highlighted some of the principles and problems associated with molecular genetic proof of a successful single parent genetic cross. Evidence of a successful genetic cross between parents of different polymorphic strains of *H. contortus* was first sought by identification of specific marker alleles in the progeny that would have been present at a high frequency in one parent, while only present at a low frequency, or absent in the other and *vice versa*. In this respect, the absence of several common MHco4 (WRS) *H. contortus* specific alleles in the *H. contortus* progenies, and the frequencies of MHco3 (ISE) and MHco4 (WRS) *H. contortus* specific alleles in the *H. contortus* progenies (illustrated in Figs 4.6 and 4.7) were wholly inconsistent with a successful single parent genetic cross.

More precise molecular analyses were based on the Mendelian principle that the frequency of any allele (*a*) in the F_1 progeny of a mating between single nematode

parents with two alleles (*a* and *b*) should be 0.75 (for an *ab* x *aa* cross), 0.5 (for an *ab* x *ab* cross) or 0.25 (for an *ab* x *bb* cross), and the frequency of heterozygotes (*ab*) in the F_1 progeny of a mating between single nematode parents with two alleles (*a* and *b*) should be 1 (*aa* x *bb*) or 0.5 (*aa* x *ab* or *ab* x *bb*). The allele frequencies in an F_2 population resulting from random mating of F_1 nematodes should be the same, and indeed the F_2 genotype frequency should be predictable on the basis of the Hardy-Weinberg law (for example, if the frequencies of *a* and *b* in the F_1 population are 0.75 and 0.25 respectively, then the genotype frequencies in the F_2 population should be 0.56 [0.75^2] *aa*, 0.38 [2 x 0.75 x 0.25] *ab* and 0.06 [0.25^2] *bb*.) The same principles apply where 3 or 4 alleles are present between the single male and female parents (Weinberg, 1909, cited by Stern, 1943). Thus the success of single parent mating can theoretically be determined from the allele and genotype frequencies of any neutral marker in the F_1 or F_2 populations. These principles have previously been outlined for the analysis of population genetic crosses to study vulval flap morphology in *H. contortus* (LeJambre, 1977).

The allele and genotype frequency data shown for both F_1 and F_2 generations are not consistent with the results expected for a successful single parent genetic cross in any case, although there were issues with failed lysates and allelic dropout. The frequency of genotyping errors due to allelic dropout and/or the genotyping of false alleles is very low (~0.016 per allele) (Redman and others, *In Press*) and would not have confounded the analyses. However, data are only shown for alleles where marker PCRs led to the generation of a useful Genescan trace. In this study, most cases of failure of the microsatellite PCRs probably resulted from poor DNA lysate template quality, but conditions in the PCR, such as the use of degraded primers, and the presence of true null alleles would also have been involved. Previous *H. contortus* population genetics studies have shown a high frequency of true null alleles in most autosomal microsatellite markers (for example at the Hcms27 locus used in this study) (Redman and others, 2008) and the presence of true null alleles is also recognised in *T. circumcincta* (Grillo and others, 2006). True null alleles probably arise due to high levels of polymorphism

in flanking sequences, as a consequence of known very high levels of population genetic diversity (Blouin and others, 1999). True null alleles may therefore indicate a definite, specific genotype and should be analysed as definite alleles (Wagner and others, 2006). While some null alleles were accounted for by repeating the PCR, it proved impossible in this study to differentiate true null alleles from poor DNA lysate template quality or insensitivity of the PCR. Null alleles could not therefore be included in the genetic analyses, and may have constrained, but not invalidated its conclusions. When genetic analyses were repeated, specifically assigning the null alleles (data not shown), they still failed to provide proof of a successful single parent genetic cross. The inability to identify true null alleles unavoidably confounds the genetic analysis of microsatellite allele data throughout this study, particularly in situations where the quality of individual nematode lysate template was poor. Nevertheless, throughout the studies presented in this thesis, genetic data proved to be crucial in determining whether or not genetic crosses had been successful.

Subjective examination of bulk microsatellite Genescan traces proved useful for the comparison of putative F_2 *H. contortus* with field isolates and laboratory maintained strains. Allele frequencies can be estimated by amplification of markers from single DNA samples derived from bulk populations of about 600 *H. contortus* L_3. This approach is based on an assumption that there is a proportionate amplification of each allele, and has been tested by comparison with individual genotypes. Bulk Genescans have been validated where the peak height is relatively large, but are less reliable for relatively small peaks (Redman and others, 2008).

3.4.2 Reproductive biology and mating patterns of *H. contortus*

3.4.2.1 The duration of fertilised egg shedding after removal of males

It has previously been stated that adult *H. contortus* females stop laying fertile eggs within 48 hours after being separated from males (Le Jambre, 1977; Le Jambre and

others 1977). However, in this study egg laying by adult MHco3 (ISE) female *H. contortus* continued indefinitely and egg hatching to L_1 persisted for 15 days after removal from males, albeit that the proportion of fertilised eggs fell to a low level from about 5 days after removal from males. Successful genetic crosses have been performed involving populations of *H. contortus* and *H. placei* (Le Jambre, 1979), or of different strains of *H. contortus* (Le Jambre, 1977; Le Jambre and Royal, 1980), having waited for 2 weeks before using eggs to ensure that they are the progeny of the imposed crosses. However, such a delay would limit the chances of a successful single *H. contortus* parent genetic cross.

The process whereby adult MHco3 (ISE) female *H. contortus* continue to shed fertilised eggs for up to 15 days after separation from males suggests that they may store sperm. In *C. elegans*, sperm are stored in a spermatheca and oocytes are fertilised in the spermatheca as they pass into the uterus (Ward and Carrel, 1979). However, while the morphology of *H. contortus* has been described, a similar structure has not been identified. Sperm storage would increase the likelihood of polyandry, which has been demonstrated to occur (Redman and others, *In Press*), leading to greater genetic diversity. Throughout this study, whenever donor or recipient sheep have been infected for a prolonged period of time, the survival rate of female *H. contortus* in their sheep host has been shown to be greater than that of males. Similar situations have been alluded to in field infections, for example during periods of drought, or in cool climates during the winter when re-infection with male L_3 may not occur. In this study, adult MHco3 (ISE) female *H. contortus* were shown to re-breed following a period of absence of males. Sperm storage could aid in *H. contortus* survival in circumstances such as the end of drought periods or early spring when young adult males are rare.

Unfertilised egg shedding by adult female MHco3 (ISE) *H. contortus* increased from day 14 after infection with MHco10 (CAVR) L_3, at which stage the males would have been late L_4 or immature adults. This increase in egg shedding, three days before fertilisation was identified, might suggest that the late L_4 or immature adult males

physically mated with the mature adult females, or that the mature adult females recognised the presence of males, raising intriguing questions about the process of attraction between small numbers of male and female *H. contortus* within a relatively large abomasum.

Demonstration of the fact that adult MHco3 (ISE) female *H. contortus* could re-breed following a period of absence of males could have been achieved by the surgical transfer of a population of male *H. contortus*. However, this would have involved subjecting the recipient lamb to a second surgical procedure. Instead, the recipient lamb was orally dosed with MHco10 (CAVR) *H. contortus* L_3 which would have included both males and females. The distinctive differences in vulval flap morphology between the MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* enabled the strain identification of adult females recovered postmortem from the abomasum of the recipient lamb to be estimated and the subsequent monitoring of fertilised egg shedding. Differences in egg shedding between the female MHco3 (ISE) and MHco10 (CAVR) *H. contortus* may have been due to inherent characteristics of the different strains. However, the higher proportion of fertilised eggs shed by the female MHco10 (CAVR) *H. contortus* might indicate that in the absence of MHco3 (ISE) *H. contortus* males, the relatively small number of MHco10 (CAVR) *H. contortus* males mated preferentially with same-aged MHco10 (CAVR) *H. contortus* females. This could have implications concerning the genetic structure of populations of mixed strains of *H. contortus*.

3.4.2.2 Egg laying by day 14 female H. contortus

Throughout this study, the minimum prepatent period (based on positive FWECs) for *H. contortus* was 17 days. Nevertheless, if genetic crosses were to be performed by the surgical transfer of day 14 late L_4/immature adult male and female *H. contortus*, then it was important to demonstrate that mating and fertilisation did not occur by this stage. This was indeed shown to be the case on the two occasions following the surgical transfer of day 14 female MHco3 (ISE) *H. contortus*. Throughout this study, the minimum prepatent periods for the MHco4 (WRS) and MHco10 (CAVR) strains of *H.*

contortus were marginally longer than 17 days, so the demonstration that fertilisation did not occur in day 14 female MHco3 (ISE) *H. contortus* would also be relevant for the other strains that were surgically transplanted in genetic crosses.

On both occasions following the surgical transfer of day 14 female MHco3 (ISE) *H. contortus*, the female nematodes once mature shed about 1,000 eggs per day, about 50% of which developed to a thin-shelled, abnormal multi-cell, blastula. Some of the eggs appeared to form an abnormally-shaped gastrula. Egg shedding was shown to involve most (at least 73%) of the females and not just a few individuals.

Many of the eggs shed by the unfertilised female *H. contortus* from both recipient lambs X and Y were heterozygous at one or more microsatellite loci. The identification of only one or two heterozygotes might have indicated a mistake in preparation of lysates, whereby two eggs were pipetted into one well of the PCR plate. However, the identification of heterozygotes in nearly half of the eggs from recipient Y showed that they were not haploid products of meiosis, but were aneuploid, diploid or polyploid. There are various possible mechanisms by which this situation could have arisen. Hermaphroditism is normal in the free-living model nematode organism *C. elegans*, but seems unlikely in the parasitic nematode *H. contortus* because sperm production has not been identified in females. Ploidy could arise from re-fusing of haploid daughter cells of meiosis. This process has been shown to occur in the absence of sperm in the plant parasitic nematode, *Meloidogyne hapla* (Liu and others, 2007). Alternatively, polyploidy might result from mitotic development of the germ cells. In *C. elegans*, meiosis is only completed once sperm penetration of the oocytes has occurred (McNally and McNally, 2005), providing a mechanism whereby the absence of sperm could trigger an alternative developmental pathway giving rise to diploid or polyploid germinal line cells. If diploidy or polyploidy arose due to a form of pre-meiotic mitosis, then a female parent that was heterozygous (*ab*) at a microsatellite locus would only shed heterozygous (*ab*) unfertilised eggs. However, if diploidy or polyploidy arose from the re-fusing of haploid daughter cells of meiosis, then the eggs could be a mixture of

homozygotes and heterozygotes (*aa*, *ab* and *bb*). The females recovered from recipient B and their unfertilised egg broods were, therefore, cryopresrved to enable such analysis at some future time.

Similar cellular division of unfertilised *Haemonchus* eggs has been described (Le Jambre, 1979; Le Jambre and Royal, 1980) in an F_1 hybrid generation produced from a genetic cross between *H. contortus* and *H. placei*, in which the males were infertile and effectively aspermic due to spermatocyte degeneration caused by a failure of chromosome pairing. This hybridisation study differed from the current study concerning the presence of males, the involvement of an F_1 *Haemonchus* hybrid rather than *H. contortus*, and the much lesser reported extent to which egg development progressed. Nevertheless, observations from the hybridisation study may help to explain the process involved in the current study. Cytological studies of the F_1 *Haemonchus* hybrids showed that the chromosomes in eggs of unfertilised females did not undergo meiosis and polar body formation, but continued to divide by a process of endomitosis, resulting in a remarkable increase in chromosome number (Le Jambre, 1979; Le Jambre and Royal, 1980). Eventually the egg cytoplasm could divide unevenly, with each new cell containing a neoplastic group of chromosomes. In common with the present study, some of the eggs were also deformed due to incomplete shell formation. It has been suggested that the genomes of *H. contortus* and of *H. placei* may have different temperature requirements (Le Jambre 1979; Le Jambre, 1980; Le Jambre and Royal 1980) and implied that the process of endomitosis may be temperature dependent. However, in the current study, incubation of unfertilised eggs at a range of temperatures did not affect the extent of cellular division that was observed.

The process of endomitosis of chromosomes is reported to be a normal occurrence in the somatic nuclei of *Trichostrongylidae* (John, 1957, cited by Le Jambre and Royal, 1980) including *Haemonchus* (Le Jambre, unpublished PhD thesis, Cornell University, 1971, cited by Le Jambre, 1979), producing a high degree of ploidy. It has been suggested that the endomitosis reported in the hybridisation study may have arisen due to the joining of

dissimilar genomes, giving rise to chromosomal duplication in the germinal line as well as normal process that occurs in the somatic line (Le Jambre and Royal, 1980). However, it now appears that a similar process occurred twice in a specific strain of *H. contortus*, indicating that the process leading to cell division in unfertilised eggs is an ancestral trait or evolutionary adaptation, rather than a genetic abnormality.

The proportions of heterozygotes at each locus were similar for eggs voided in the faeces of both recipient lambs infected only with day 14 female *H. contortus* (albeit that only a small number of eggs from recipient lamb A were genotyped). No heterozygotes were identified at the Hcms 22co3 and 18210 microsatellite loci, despite these loci being polymorphic. Furthermore, the heterozygosities that were observed at different loci (H_o) differed to different extents (H_e/H_o) from the heterozygosities that would have been expected to have arisen if mitotic division of the oocytes had occurred uniformly (H_e). Together these observations imply that if endomitosis did occur, the replication of the 6 chromosome pairs was not uniform, but the pattern of variation between chromosomes was consistent. Furthermore, if endomitosis occurred to different extents in different chromosomes, then it might have influenced the relative amounts of DNA lysate template derived from different chromosomes, accounting for differences in the efficiency of PCR that could have led to a higher frequency of null alleles for loci on particular chromosomes. This could be investigated by examining metaphase spreads of the embryos.

Some of the unfertilised eggs shed in the faeces of recipient Y appeared to undergo some degree of cell migration or morphogenesis (for example Fig 3.14, E and F). This would be unlikely to have arisen simply from random uneven cell division (Le Jambre, 1979; Le Jambre and Royal, 1980). The process of endomitosis is not dissimilar from that of mitotic parthenogenesis, and the observation of gastrulation in unfertilised eggs might indicate partial parthenogenetic development. The biotic potential of *H. contortus* is extremely high (Coyne and Smith, 1992), and the possibility exists that under specific conditions such a trait could evolve and that it could then prove to be advantageous.

3.4.2.3 Studies of interbreeding between different strains of H. contortus

Throughout this study, the patterns of faecal egg counts have differed between donor lambs infected with MHco3 (ISE), MHco4 (WRS) or MHco10 (CAVR) strains of *H. contortus*. Some of this variation would have been due to factors such as the age and immune status of the donor lambs, the time of year, or the age of the L_3 used to generate the infections. However in setting up the donor lambs for the mixed infection experiments, the same-aged donor lambs were infected with the same numbers of similar-aged L_3 on the same day and different trends in FWECs were still observed. This suggests there may be phenotypic differences in life history traits between the isolates. Furthermore, the FWECs of the recipient lambs receiving pairwise mixed infections were intermediate between those observed for the two parent strains with which they were infected lending weight to the assumption that differences in FWECs reflect inherent differences in the reproductive behaviour of the different strains of *H. contortus*. Female *H. contortus* of different strains may reach maturity at different stages, may mate with different numbers of males with different frequencies, and/or may show differences in egg production. It has been suggested that such inherent differences give rise to a weakness of mutual attraction between males and females of different closely related species such as *H. contortus* and *H. placei* in a mixed infection (Bremner 1956), but that in the absence of conspecific partners, the closely related species can freely interbreed (Le Jambre, 1979). Genetic analysis using microsatellite markers has shown that genetic fingerprints of the MHco3 (ISE), MHco4 (WRS) or MHco10 (CAVR) strains of *H. contortus* differ and that the MHco3 (ISE) and MHco10 (CAVR) strains are extremely divergent. It has been shown that similar levels of genetic diversity reflect cryptic speciation in *T. circumcincta* (Grillo and others, 2006).

Mixed infection experiments were set up to investigate differences in the life history traits of the different isolates and the extent to which they will interbreed in the presence of equal numbers of conspecific partners. The PCA plots suggest that breeding occurred freely between and within either MHco3 (ISE) and MHco4 (WRS), or MHco4 (WRS)

and MHco10 (CAVR) strains of *H. contortus* in mixed infections. In contrast the F_1 progeny of the MHco3 (ISE) and MHco10 (CAVR) mixed infection were predominantly MHco3 (ISE) genotypes with a smaller number of hybrid multilocus genotypes suggesting some inter-strain breeding. Thus, while the MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* appear to freely interbreed in the absence of conspecific partners (Chapter 5), the two genetically divergent isolates of the same species appear not to freely interbreed in a mixed infection situation. Indeed it appears that the MHco3 (ISE) strain outcompetes the MHco10 (CAVR) strain when present as a mixed infection. One alternative explanation is that the results reflect the possibility that the prepatent period of the MHco3 (ISE) strain might be shorter than that of the MHco10 (CAVR) strain.

The genetic analysis using PCA plots was limited by the number of markers, and possibly also by missing data due to poor amplification from certain lysates. The microsatellite PCAs have since been repeated using the same lysates of day 28 eggs with an additional marker (Hcms36) and four new markers (3086A, 22193Y, 44104 and 53265) (Fiona Whitelaw, *Personal communication*). The plots suggest that the MHco3 (ISE) strain of *H. contortus* out-competes both the MHco4 (WRS) and the MHco10 (CAVR) strains in mixed infections, but that the MHco3 (WRS) and MHco10 (CAVR) strains together breed freely (Fig 3.40).

This study investigated the population genetic structures of the progeny of different strains of *H. contortus* produced on day 28 following L_3 infection. *H. contortus* of each of the parental strains would have reached maturity by this stage, despite the differences in egg production, but it is possible that differential maturation rates between different strains could influence their freedom of interbreeding. Lysates were therefore made of the progeny produced on days 21 and 36 and we intend to follow up this work by genotyping F_1 larvae harvested at later stages of mixed infection starting with day 36. Individual *H. contortus* recovered postmortem from the abomasa of the donor lambs and

their L_1 broods were stored in absolute ethanol to enable further investigation of whether or not individual females of one strain preferentially mate with males of the same strain.

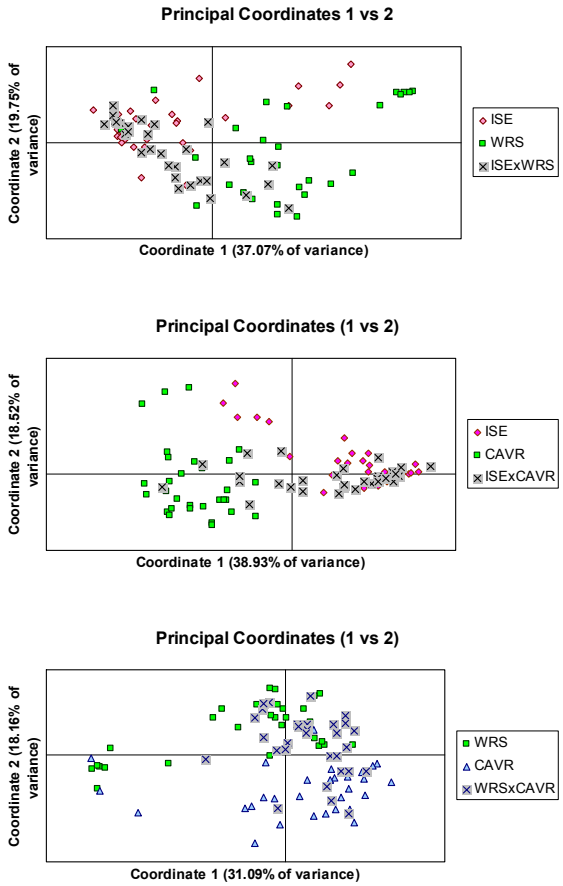


Fig 3.40: Additional principal coordinates analysis plots (courtesy of Fiona Whitelaw, Glasgow University Veterinary School) for MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* and the progeny of co-infection experiments using mixtures of two different strains using a panel of 11 microsatellite loci. Each point represents an individual nematode.

Knowledge of whether or not different strains of *H. contortus* interbreed freely is required in order to understand the population genetics of the parasite. This understanding will be a prerequisite for the use of molecular markers to study the genetics of anthelmintic resistance, once such markers are identified. For example, if a rarer strain of *H. contortus* carrying alleles of genes conferring anthelmintic resistance is

introduced to a mixed infection, the probability of an individual finding a mate of the same species would be the square of the proportion of its strain in the total infection (Le Jambre, 1979; Le Jambre 1981). Therefore the rarer strain will suffer serious population loss to the point of extinction of the anthelmintic resistance genes if it does not interbreed. Furthermore, the proportion of the anthelmintic resistance alleles in the total population will be governed by the extent to which interbreeding freely occurs. Different strains of *H. contortus* have also been shown to differ in other respects, such as the temperature requirements for larval development (Le Jambre, 1981), which could further influence the population genetics of introgression of anthelmintic resistance alleles.

3.4.2.4 Effects of environmental conditions affecting molecular marker allele frequencies of non-parasitic stages of Haemonchus contortus

Some L_3 are used within a few weeks of collection while others may be stored at 10^{o}C for up to 2 years, by which time significant proportions have died, before the population is used to infect a donor sheep. L_3 survival is probably randomly determined by environmental factors, such as fungal or bacterial contamination, but could also be influenced by genetic factors determining fitness and longevity. If this is the case, and these genes are linked to genes conferring anthelmintic resistance, then environmental conditions affecting free living parasitic stages could influence the population genetics of anthelmintic resistance.

While the bulk Genescan traces for five microsatellite loci for L_3 cultured and stored under different conditions were more-or-less indistinguishable from each other, this information was inconclusive, because the likelihood of any of such a small panel of markers being linked to genes conferring fitness or longevity would have been small. The experiment nevertheless showed that the marker profiles were robust over time and illustrated an application for the interpretation of bulk Genescan traces. In a similar manner, bulk genescan traces have been consistently demonstrated not to change over

the course of an infection (Libby Redman, *personal communication*; *Data collected in the preparation of this thesis*).

3.4.3 Phenotypic characterisation of isolates and strains of *H. contortus*

3.4.3.1 The larval feeding inhibition assay

Drug concentrations at which 50% of the L_1 fed (LFI_{50} values) could not be determined in this study. (This was due to the fact that larval feeding in ivermectin-free control replicates was generally insufficient to generate LFI_{50} values. This problem could have been addressed by correcting the data for 100% feeding in the drug free control replicates as is sometimes practised in the EHA (Georg Von Samson-Himmelstjerna, *examiner's comment*), but this would have introduced large errors, which would have been inconsistent between the different populations being investigated.) However, the LFIA was able to detect differences in the proportion of L_1 that were able to feed at higher drug concentrations between anthelmintic resistant and susceptible strains. Hence, comparison of strains at the LFI_{90} and LFI_{99} discriminatory drug doses allowed the identification of resistant strains of *H. contortus*. This principle has been applied to a larval development test for ivermectin resistance, in which the LD_{50} values for ivermectin resistant isolates were only two-fold higher than those of susceptible isolates, but a small proportion of the resistant isolates developed at significantly higher drug concentrations than susceptible isolates (Kotze and others, 2002).

The LFIA did not prove to be particularly useful for the quantitative determination of the ivermectin resistance phenotype because the LFI_{90} and LFI_{99} discriminatory drug concentrations produced varied greatly within strains of *H. contortus* with the same *in vivo* resistance phenotype. However, subjective analysis of dose response curves for LFIAs that were started at the same time using L_1 hatched from eggs voided by similar-aged and similarly managed donor sheep was useful for the comparison of ivermectin susceptible and resistant strains of *H. contortus*, albeit that the differences in

discriminatory drug doses between resistant and susceptible strains were less than 2 fold. Previous studies reported much higher LFI_{50} values (about 0.1 and 8 µg/ml ivermectin for Hco3 and Hco4 *H. contortus* respectively) (Álvarez-Sánchez and others, 2005), which were about ten-fold higher than LFI_{90} or LFI_{99} values that were calculated during the current study. While the reason for this difference is unclear, these studies also identified large differences in LFI_{50} values throughout the course of donor infection, and concluded that the LFIA was most useful when used for the comparison of ivermectin resistant and susceptible nematode strains (Álvarez-Sánchez and others, 2005).

Throughout the winter months, L_1 feeding in drug-free controls was poor and associated fungal contamination of the assays was prominent. This problem was not satisfactorily addressed by manipulating the conditions involved with egg extraction and incubation of the assays. Fungal contamination would have originated both within the sheep host and as airborne spore contamination of the environment in which the assays were set up. However, while many fungal species such as *Duddingtonia flagrans* are known to be nematophagous and have been considered as candidates for biological control (Jackson and Miller, 2006), it is more likely that the fungi were opportunistic contaminants of L_1 that did not feed, rather than the primary cause of poor L_1 feeding in the LFIAs.

In vitro methods to determine anthelmintic resistance are more manipulable than *in vivo* methods, because they can be used to produce dose response curves. This may be useful if, there are several genes for resistance rather than a single gene, perhaps involved at different drug concentrations. However, *in vitro* bioassays can only provide information about the mechanisms of anthelmintic resistance involved with the specific assay, for example the LFIA primarily involves pharyngeal pumping in L_1. The nervous control of pharyngeal pumping in L_1 and the way in which ivermectin is presented in the LFIA probably differ from the pharmacology and effects of ivermectin in parasitic nematode stages in their sheep host. Furthermore, poor levels of feeding in drug-free controls shows that pharyngeal pumping in L_1 is inhibited by factors other than ivermectin, such as the general fitness of the L_1. The genetic basis of the *in vitro* ivermectin resistance

phenotype measured in the LFIA may therefore differ from the genetic basis of ivermectin resistance *in vivo*.

3.4.3.2 The effects of donor age and immunity

The study of the effects of donor sheep age and immune competence on host parasitological aspects of *H. contortus* infection was conceived in an attempt to explain the reasons for the poor success of nematode infections of donor and recipient sheep used for genetic crossing studies during the winter months. From the outset, it was intended only to provide insight into possible reasons for the problem. It was recognised from the outset that the fact that only one lamb, two control hoggs and two immune hoggs were infected with each *H. contortus* isolate would prevent valid statistical analysis. The use of sufficient animals to provide statistically valid results would have been impractical and inappropriate. The effects of sheep age were examined by comparing 3 - 4 month-old worm-free donor lambs, with 14 – 15 month-old hoggs that had been reared under similar worm-free conditions. Therefore, while the hoggs would not have acquired specific immunity against nematode parasites, they would have acquired age-related immunity against a variety of non-specific antigens that would have been expected to influence their immune response to *H. contortus*. Suppression of immunity by weekly injections of 1.3 mg/kg of methyl progesterone acetate was a crude approach that might have been expected to result in other confounding host physiological disturbances. However, the method was chosen because the same corticosteroid treatment regime has been used to immune suppress 5 month-old naïve lambs and 17 month-old sheep that were dosed daily with *T. colubriformis* L_3 (Greer and others, 2005a). In this report, the success of immune suppression was based on 3-fold higher FWECs, comparative worm burdens and higher ELISA titres for *T. circumcincta* specific total antibodies in the corticosteroid treated sheep compared to untreated sheep in the same cohorts (Greer and others, 2005a). Similar effects of immune suppression against abomasal nematode parasites have also been reported (Greer and others, 2005b).

Variations in immune responsiveness and subsequently the size of the abomasal nematode parasite burden within different age classes of sheep are well established (Reid and Armour, 1975a; Watson and others, 1986; Hohenhaus and others, 1995). The differences that were seen in the extent and patterns of FWECs between the 3 - 4 month-old lambs and 14 – 15 month-old hoggs, were therefore as expected. However, unlike previous studies (Greer and others, 2005a; Greer and others, 2005b) no differences in FWECs were seen between the corticosteroid immune suppressed hoggs and control animals. This contradictory result probably arose because the previous study (Greer and others, 2005a) investigated the effects of age and immune suppression of the hosts' response to daily exposure to infective larvae, while the current study involved a single dose of infective larvae. Current understanding of the cell mediated immune response in the gastrointestinal epithelial tissue is rudimentary, but it is likely that different mechanisms are involved in resistance to larval establishment and in performance in the face of established infection (Sykes and Coop, 2001).

Unlike previous studies (Greer and others, 2005a), no significant differences were seen between the total abomasal *H. contortus* burdens of the 4 month-old lambs, 15 month-old hoggs, or corticosteroid immune suppressed 15 month-old hoggs infected with the MHco3 (ISE), MHco4 (WRS) or MHco10 (CAVR) strains of *H. contortus*. However, adult female *H. contortus* in the abomasa of 4 month-old lambs shed 6.5, 3.1 and 8.0 times more eggs than adult female *H. contortus* in the abomasa of 15 month-old hoggs infected with MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* respectively. Adult female *H. contortus* in the abomasa of the corticosteroid immune suppressed 15 month-old hoggs shed 1.5, 2.1 and 1.9 times more eggs than adult female *H. contortus* in the abomasa of the untreated 15 month-old hoggs infected with MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* respectively. Thus, while host immunity did not have any effect on the number of *H. contortus* surviving in the abomasum, it had a profound effect on egg shedding by individual female nematodes. Furthermore, this effect varied between different strains of *H. contortus*, which could influence the population genetics of mixed infections. If host immunity can influence

the number of eggs shed by adult female nematodes (for example, Strain and others, 2002) then it is conceivable that it could also influence the subsequent development of those eggs (Jørgensen and others, 1998). Experiments which compare the development of free living parasite stages cannot, therefore, assume that eggs taken from different donor sheep will have comparable developmental success.

The comparative study examining the effects of sheep age and immune competence on the results of bioassays for anthelmintic resistance was conceived to investigate possible reasons for poor L_1 feeding in LFIAs set up during the winter months. Variation has been shown between hosts of different ages in the developmental success of *T. circumcincta* eggs, with significantly greater rates of development to L_3 of eggs voided by young lambs and reduced viability of eggs voided by adult ewes (Jørgensen and others, 1998). These findings are consistent with an immune mechanism acting on nematode parasitic stages within the host influencing free living stages outside the host (Jørgensen and others, 1998), and could provide a possible explanation for the poor L_1 feeding in LFIAs using eggs shed by older, immune competent donor sheep. However, neither the age of the donor sheep, nor corticosteroid immune suppression had any effect on egg hatching or larval feeding at different concentrations of anthelmintic drugs in bioassays in this study. Thus, the poor level of L_1 feeding in LFIAs set up during the winter months was probably not caused by the age and immune competence of the donor sheep.

The previous studies evaluating the LFIA for the determination of the ivermectin resistance phenotype would support the premise that mechanisms acting on nematode parasitic stages within the host could influence the developmental success of free living stages. Older worm-free donor animals used during the winter months would always have been housed and fed a diet of conserved forage and concentrates that would have contained relatively low levels of molybdenum for a period of 8 to 14 months. Under these conditions, accumulation of copper within the liver would have been inevitable, potentially leading to toxicity problems (Sargison, 2001). In fact, very high copper

concentrations were identified in December 2007 in a group of surgical transfer donor lambs, from which disappointingly insufficient numbers for purpose of *H. contortus* were recovered. The liver and kidney copper concentrations of these lambs were 6370, 8160 and 3350 μmol/kg and 249, 320 and 296 μmol/kg respectively, consistent with copper accumulation, and in one animal indicating chronic copper toxicity (liver and kidney copper concentrations > 7850 and 314 μmol/kg). Copper is known to have direct anthelmintic properties against abomasal parasites reducing their establishment (Bang and others, 1990), but the effects of exposure of eggs shed by adult female parasites to high levels of copper on larval feeding and development are unknown. While unproven, it is possible that high copper levels acting on nematode parasitic stages within the sheep host could have influenced the developmental success of free living stages.

An alternative mechanism acting on nematode parasitic stages within the host that might influence the developmental success of free living stages during the winter months could be related to daylength. Changes in daylength influence the secretion of melatonin from the pineal gland of sheep. Melatonin secretion increases in response to declining day length from mid summer and is reduced with increasing daylength from mid winter. Consequently, the amounts and frequency of release of gonadotrophic hormones changes over the winter months. It is possible that these hormones could influence the reproductive behaviour of parasitic nematodes, and that such effects could vary between strains, depending on the latitude at which they developed. (For example, the MHco3 (ISE) strain of *H. contortus* was thought to have originated from equatorial Kenya and the MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus* originated at between 20 and 30 degrees south. It is possible that the poor development of free-living stages of these strains of *H. contortus* could be related to a failure to adapt to the pattern of daylength at 57 degrees north at the Moredun Research Institute, although this was not investigated during the current study.)

3.4.3.3 Dominance of the resistance phenotype

The post ivermectin treatment efficacies for the F_1 progeny of a genetic cross between the ivermectin susceptible MHco3 (ISE) and ivermectin resistant MHco4 (WRS) strains of *H. contortus* were 98% following the use of a full therapeutic dose rate of 200 µg/ml of ivermectin and 0% following the use of a half therapeutic dose. These results must be interpreted with caution because they were derived from only two sheep and could have been influenced by differences in host physiology affecting the pharmacokinetics of the anthelmintic drug. Use of a discriminating half dose of ivermectin has been proposed as a method to improve the sensitivity of the undifferentiated faecal egg count reduction test (Palmer and others, 2001) and has been adopted in the field for the diagnosis of macrocyclic lactone resistance (Lawrence and others, 2006). However, underdosing lowers the test's specificity (Van Wyk and others, 1997b) and may identify resistance arising due to different mechanisms. It is therefore likely that the diagnosis of ivermectin resistance based on the post treatment efficacy of a half dose of ivermectin, but demonstration of ivermectin susceptibility to a full dose of ivermectin (albeit that a few resistant *H. contortus* survived treatment), implies the presence of more than one mechanism of resistance to ivermectin *in vivo*. The results might imply that the mechanism involved with resistance to the half dose of ivermectin is partially dominant, while the mechanism conferring resistance to the full dose of ivermectin could be partially recessive. However, these analyses are based on unproven and likely to be incorrect assumptions that the parent population genotypes were monomorphic and homozygous at loci conferring resistance to each mechanism. Furthermore, heterozygotes may survive underdosing with a half dose of ivermectin, even if the resistance trait is partially recessive (Besier and Hopkins, 1988).

Based on Mendelian principles, the results of this study suggest that the *H. contortus* benzimidazole resistance phenotype in the EHA is recessive, while the ivermectin resistance phenotype in the LFIA shows partial dominance. These results broadly agree with those of published studies of the genetic basis of ivermectin resistance (for example, Dobson and others, 1996; Roos, 1997; Le Jambre and others 1979; Le Jambre

and others, 2000). However, the application of Mendelian genetic principles is based on knowledge or assumption that the genotypes of individuals in the parental population are monomorphic, and that a single mechanism of resistance is involved. Neither assumption is likely to be correct and it is likely that the resistance phenotype in bioassays is due to several different genotypes and that these differ from genotypes conferring anthelmintic resistance *in vivo*. If a single locus was involved, then inflections would have been expected in the dose response curves for the bioassays for the F_2 populations corresponding with 25% and 75% indicating a 1:2:1 segregation of genotypes (Le Jambre and others 1979a). The absence of such inflections therefore implies a polygenic basis of resistance.

The isotype 1 β tubulin F200Y genotypes of both the benzimidazole susceptible MHco3 (ISE) and the benzimidazole resistant MHco4 (WRS) parental populations were polymorphic. The F200Y SNP allele frequencies of 0.95*T*: 0.05*A* determined throughout this study were similar to the published F200Y allele frequencies of 0.97*T*: 0.03*A* for the same strain of *H. contortus* based on a real time PCR method (Walsh and others, 2007). The fact that 18 of 25 L_1 that hatched in a concentration of 0.1µg/ml of thiabendazole in the EHA were homozygous *TT* implies that mechanisms in addition to the isotype 1 β tubulin F200Y SNP mutation are involved in the *H. contortus* benzimidazole resistance phenotype in the EHA, supporting the conclusions of previous studies on *T. circumcincta* (Stenhouse, 2007).

Thus, determination of the anthelmintic resistance phenotype to the accuracy that would be required for comparison with the genotype of the progeny of a single parent genetic cross aimed at identifying molecular markers for ivermectin resistance may prove to be impossible.

3.5 Chapter summary with reference to the development of genetic crossing methods to identify molecular markers for ivermectin resistance

Phenotypic and genotypic analysis of the F_1 and F_2 progeny of a genetic cross between single ivermectin susceptible and ivermectin resistant parent nematodes would provide a logical basis for genetic approaches and genome wide comparisons to identify potential candidate genes for resistance. Unfortunately, the likelihood of successfully producing and providing genetic confirmation of a genetic cross simply by placing a single male and female nematode into the abomasum of a recipient sheep is low. The preliminary attempts to provide proof of concept of a single nematode parent genetic cross were disappointing, but they highlighted certain issues that need to be addressed in order to develop a genetic crossing method to identify candidate genes. The issue of parasitic nematode contamination of donor sheep and of coprocultures can be addressed, for example by developing a method that uses filial eggs within 17 days surgical transfer, and by conducting coprocultures in a closed environment. The recognition of coprophagous insects as nematode parasite vectors raises challenging questions about their role in gene flow and nematode population dynamics. Although there is a limited number of molecular markers and there are problems associated with null alleles, this work is a strong proof of concept in both the need to validate apparently successful crosses genetically and the value of the markers in doing so. Concerns about microsatellite markers are being addressed as new, robust, polymorphic loci are identified, but there is now a need for greater understanding of the population genetics of nematode parasites. The value of microsatellite Genescan traces derived from PCRs of bulked DNA lysates was demonstrated for the comparison of different parasitic nematode populations.

A greater understanding of parasitic nematode reproductive biology is a prerequisite for the development of a genetic crossing method, to provide proof of success of a genetic

cross, and for the application of molecular markers to the practical study of anthelmintic resistance in sheep flocks. Previously poorly understood aspects of the reproductive biology of *H. contortus* were investigated, such as spermatozoal storage by adult females, and the shedding of polyploid eggs in the absence of fertilisation. Further study is now needed to elucidate the influence of these mechanisms on genetic analyses. These preliminary studies consistently highlighted inherent parasitological differences between strains of *H. contortus*, raising important questions concerning the population dynamics and genetics of mixed infections. Although the co-infection experiments are incomplete, they demonstrate the development of an approach whereby the behaviour of two nematode parasite isolates infecting the same host can be compared to investigate the extent to which they interbreed.

Accurate determination of the anthelmintic resistance phenotype will be required for the genetic analysis of a single parent genetic cross. *In vivo* methods to identify and recover anthelmintic susceptible nematodes have not been developed and may prove to be impracticable. While the EHA provides a reliable bioassay for the diagnosis of benzimidazole resistance, its accuracy in determining the anthelmintic resistance phenotype of an individual organism is compromised by the likely contributions of multiple genetically controlled biochemical mechanisms of benzimidazole resistance. The LFIA proved to be inconsistent for the *in vitro* diagnosis of ivermectin resistance, especially during the winter months, but was nevertheless useful as a comparative tool to characterise different parasitic nematode strains and populations. In this regard, subjective comparison of dose response curves proved to be more informative than analysis of discriminatory drug dose concentrations. Furthermore, application of the LFIA to the determination of the resistance phenotype of individual nematodes will almost certainly be confounded by the existence of multiple mechanisms, involving both drug receptors and drug regulatory mechanisms and by a stochastic element that is not fully understood. Similar considerations also compromised the ability to reliably determine the dominance of the anthelmintic resistance phenotype. Some of the problems associated with the use of bioassays can be addressed by confining genetic

crossing experiments to young lambs during the spring and summer months, but their inability to accurately determine the anthelmintic resistance phenotype constrains the value of a single nematode parent genetic crossing method to identify potential candidate genes for resistance.

Study of the reasons for poor feeding in the LFIA led to the concept that factors affecting adult parasitic nematodes within their host could influence the development of free living stages. While these factors were not identified, consideration of this possibility could have consequences for the study of population genetics. An effect of the sheep host age and immunity was shown on the number of *H. contortus* eggs shed by individual female worms. Understanding of factors such as these will prove to be important once molecular markers for anthelmintic resistance have been found and will require modelling.

These preliminary studies identified shortcomings of the single nematode parent genetic crossing method and identified the need to develop an alternative genetic crossing method as a basis for genome wide comparisons to identify genes associated with macrocyclic lactone resistance.

Chapter 4: Inbreeding of the MHco 3 (ISE) strain of *Haemonchus contortus*

4.1 Introduction

The previous chapter showed that a successful single nematode pair mating could not be validated following the surgical transfer of single male and female *H. contortus* directly into the abomasum of a recipient lamb. Indeed, there are no reports in the scientific literature to-date of successful single pair matings of strongylid nematode parasites that have been validated by molecular genetic characterisation. The work presented in the previous chapter demonstrated that such validation is critical as the appearance of strongylid eggs in the faeces of host recipient animals is insufficient in itself to prove success of a single nematode pair mating. The aims of this chapter were: to develop an alternative method to enable recovery of the progeny of single *H. contortus* parents; to provide unambiguous parasitological and molecular proof of successful single pair mating; and to use the method to inbreed the MHco3 (ISE) strain of *H. contortus* in a genetically validated manner.

One of the limiting factors to the success of the single pair matings attempted in the previous chapter was the potential difficulty of two single nematodes finding each other in the abomasum of a sheep. One way to increase the probability of mating might be to transplant about 20 adult female nematodes along with a single male. In this way several female nematodes could be recovered on autopsy in the hope that at least one would have successfully mated. Since only a single male would have been present, then any female nematode recovered on autopsy could only have mated with that single male. This experimental design is important since mating in *H. contortus* is polyandrous, so if any more than a single male was placed in the abomasum there would be a risk of female nematodes mating with more than one male.

4.1.1 Applications for inbred strains of *Haemonchus contortus*

4.1.1.1 Genome sequencing project

H. contortus is an important model parasitic nematode system, and a fully annotated genome is a prerequisite to apply functional genomic approaches to study basic parasite biology, identify drug and vaccine targets and understand processes such as the evolution of anthelmintic resistance at the molecular level. A sequencing and mapping project for the *H. contortus* genome has been undertaken by the Pathogen Sequencing Unit of the Wellcome Trust Sanger Institute. This project has generated a variety of resources including: over 600 megabases of shotgun sequence (representing about 6-fold genome coverage, assuming a genome size of 100 mb); bacterial artificial chromosome (BAC) libraries (with 13 BAC inserts fully sequenced); and fosmid libraries. However, assembly of the genome into large contigs and scaffolds has been problematic due to an extremely high level of sequence polymorphism between individual worms within populations of the inbred MHco3 (ISE) *H. contortus* strain (John Gilleard, *personal communication*). The best assembly of the current shotgun sequence has a median contig size of 2.8 kb, which is now assembled into larger scaffolds using read pair information (John Gilleard, *personal communication*). The genome sequencing project has used DNA template produced from populations of the inbred MHco3 (ISE) strain of *H. contortus*, but as more genetic characterisation has been performed, it has become evident that this strain remains highly polymorphic. One possible solution to the problem of genome assembly could be afforded by the development of an inbred, near-isogenic MHco3 (ISE) line of *H. contortus* from the progeny of single female and male parents that could be used for DNA template preparation prior to whole genome sequencing. This would rescue the levels of polymorphism in the genome sequence generated.

4.1.1.2 Genetic mapping

In the absence of an assembled and annotated *H. contortus* genome, the position of neutral markers, for example linked to the anthelmintic resistance phenotype, cannot yet be determined and analysis to identify linkage to candidate genes is not possible. An alternative approach to enable linkage analysis could be afforded by the creation of a genetic map (Le Jambre and others, 1999b). Genetic mapping approaches in *H. contortus* have a number of different potential applications. For example, they could be used to provide long range genetic landmarks to aid the process of genome assembly. Genetic mapping approaches could also be used to investigate the genetic basis of resistance and identify regions of the genome that are linked to a resistance-conferring mutation (Grant, 2001; Pritchard, 2001). Genetic mapping studies require nematode strains that have minimal within strain polymorphism, but high levels of between strain polymorphism, in order to allow the alternative parental alleles to be identified in F_2 progeny resulting from a genetic cross. At present, the available laboratory strains of *H. contortus* are too polymorphic to use in conventional mapping studies, and so there is a need to derive inbred, near-isogenic strains. The fact that there are high levels of genetic differentiation between the characterised ivermectin susceptible MHco3 (ISE) strain and ivermectin resistant strains of *H. contortus* such as MHco4 (WRS) and MHc10 (CAVR), means that if inbred strains can be derived from these, they would be ideal for genetic mapping studies to investigate the genetic basis of resistance.

4.1.2 The MHco3 (ISE) strain of *Haemonchus contortus*

The original ISE strain of *H. contortus* was inbred from a highly heterogeneous (Otsen and others, 2000b; Otsen and others, 2001), out-bred population by dissecting the eggs from an adult female SE strain *H. contortus*, culturing these eggs for 7 days in worm free faeces, and injecting recovered L_3 into the rumen of a recipient sheep. The recipient sheep had been killed one week after they had started shedding trichostrongyle eggs. A single benzimidazole susceptible adult female *H. contortus* had then been selected on the

basis of its β-tubulin isotype 1 genotype, and the process repeated fifteen times (Roos and others, 2004), to yield an inbred benzimidazole susceptible isolate. A cryopreserved aliquot of L_3s of this isolate was obtained from Dr Fred Borgsteede (Central Veterinary Institute, Lelystad, Netherlands) by Dr John Gilleard (Glasgow University Veterinary School) and subsequently maintained at the Moredun Research Institute by serial passage through donor sheep. The Moredun stock of this isolate was renamed MHco3 (ISE) in order to distinguish it from other versions of the ISE strain that may be used in other laboratories and to definitively designate this version as the isolate used for the genome sequence. However, genetic analysis with microsatellite markers has revealed high levels of genetic polymorphism in the MHco3 (ISE) strain of *H. contortus* (Redman and others, 2008). Furthermore, despite selection from females with susceptible β-tubulin isotype 1 genotypes, the single amino acid substitution from tyrosine to phenyalanine at position 200 (F200Y) of the polypeptide encoded by the isotype 1 β-tubulin gene (Kwa and others, 1994) is present in about 5% of the MHco3 (ISE) population (Walsh and others, 2006; Chapter 3). At first sight this high level of polymorphism of a strain that had undergone 15 rounds of serial inbreeding by taking progeny from a single female worm seems surprising. However recent work has shown that mating of *H. contortus* is polyandrous with single females mating with at least 4 different males (Redman and others, *In Press*). Consequently, simply passaging from the progeny of single female worms obtained from standard experimental infections is unlikely to be an efficient approach to inbreeding.

4.2 Materials and methods

4.2.1 General inbreeding parasitology

All of the worm-free donor and recipient lambs that were used in this study were treated sequentially with 5 mg/kg of fenbendazole (Panacur 2.5%; Intervet) and 7.5 mg/kg of levamisole (Levacide 3%; Norbrook) 7 to 14 days before oral infection with L_3 or surgery.

4.2.1.1 Surgical transfer of one male and multiple female H. contortus

A 4 month-old worm free donor lamb was orally dosed on day 0 with about 10,000 MHco3 (ISE) *H. contortus* L_3. The donor lamb was euthanased on day 14 post infection and the contents of its abomasum were collected. A single sexually immature male and 32, 20 and 20 sexually immature day 14 females were then surgically transferred within about 2 hours of recovery from the donor lamb into the abomasa of 4 month-old recipient lambs A, B and C respectively. The FWECs of the three recipient lambs were monitored daily during the following week (days 14 – 21) and supportive evidence of the identity of eggs as those of *H. contortus* was sought by fluorescent agglutinin staining (Palmer and McCombe, 1996).

4.2.1.2 Collection of progeny from single female H. contortus *following mating with a single male*

The three recipient lambs were euthanased 7 days after surgical transfer (day 21). Any *H. contortus* that were found within their abomasa were immediately picked into sterile PBS and then transferred individually into 1 ml of warm RPMI tissue culture medium in the wells of 24 well plates and incubated in 5% CO_2 at 37^{o}C for 8 hours to promote egg shedding. The 24 well plates containing adult female *H. contortus* and their egg broods were then transferred to a 24^{o}C incubator and left for 36 hours to permit hatching of any fertilised eggs.

The adult female MHco3 *H. contortus* and their F_1 L_1 broods were arbitrarily named (N1 was recovered from recipient lamb A and N2, N3 and N4 were recovered from recipient lamb B). DNA lysates were prepared from about half of the F_1 L_1 in each brood and from the head of each adult female *H. contortus*. The remaining L_1 were then transferred onto a disc of cotton filter paper placed in a petri dish containing about 5 ml of a culture of OP50 *Escherichia coli* in LB/streptomycin broth (N1, N3 and N4), or inoculated into 10 g of faeces collected from a worm-free donor lamb that had been sequentially treated with 5 mg/kg of fenbendazole and 7.5 mg/kg of levamisole 5 days previously (N2). The filter paper/*E. coli* and faecal larval cultures were then placed individually in perforated plastic bags and incubated in a closed laboratory incubator at 24^{o}C for 7 days. L_3 were then recovered from the larval cultures by Baermannasation and transferred in tapwater into tissue culture flasks and stored at 8^{o}C for 3 weeks before they were used to infect donor lambs. The terminology used in this chapter to describe the inbred lines is summarised in Table 4.1.

	Inbred line derived from recipient lamb A	**Inbred line derived from recipient lamb B**
Adult female *H. contortus* recovered on autopsy	N1	N2
First filial population	MHco3.N1 F_1	MHco3.N2 F_1
Second filial population	MHco3.N1 F_2	MHco3.N2 F_2
Third filial population	MHco3.N1 F_3	MHco3.N2 F_3
Fourth filial population	MHco3.N1 F_4 MHco3.N1 F_4 (2)*	

* propagated by infection of a second donor lamb, that was different from the donor lamb used to propagate the MHco3.N1 F_4 population

Table 4.1: Terminology used for the female parents and subsequent inbred *H. contortus* lines.

4.2.1.3 Propagation of inbred lines of MHco3.N1 and MHco3.N2

A 6 month-old worm-free donor lamb was infected with about 15 L_3 that had been cultured from F_1 (MHco3.N1 F_1) L_1 hatched from eggs shed by female N1 and a 7 month-old worm free donor lamb was infected with about 20 L_3 that had been cultured

from F_1 (MHco3.N2 F_1) L_1 hatched from eggs shed by female N2. Harnesses and faecal collection bags were fitted to both donor lambs from 14 days after L_3 infection to enable faecal collection and coproculture of the F_2 progeny. This process was repeated, infecting 8 – 9 month-old worm-free donor lambs with about 7,500 MHco3.N1 F_2 L_3 and about 7,500 MHco3.N2 F_2 L_3 to produce F_3 populations; and infecting a 10 month-old donor lamb with about 7,500 MHco3.N1 F_3 L_3 to produce an F_4 population. FWECs of the donor lambs and egg development were routinely monitored. Support of the identity of the eggs as *H. contortus* was periodically sought by fluorescent agglutinin staining and confirmation of the species identity of L_3 was periodically sought by examination of larval morphology and using ITS2 and NTS molecular markers. The donor lambs were maintained for between 2 and 5 months before they were euthanased and *H. contortus* were recovered from their abomasa, counted and stored in absolute ethanol. Individual DNA lysates were prepared from the heads of MHco3.N1 F_2 and MHco3.N2 F_1 adult *H. contortus*, and the remaining nematodes were stored in absolute ethanol. DNA lysates were made from both individual and bulk preparations of about 500 L_3 from each filial generation.

4.2.1.4 Study of egg development and hatching

Another three month-old worm-free donor lamb was infected with about 5,000 MHco3.N1 F_3 L_3. A harness and faecal collection bag was fitted from 14 days after L_3 infection to enable faecal collection, FWECs and coproculture of MHco3.N1 F_4(2) L_3.

Development and hatching of the MHco3.N1 F_4(2) eggs were monitored between days 14 and 36 by incubating aliquots of about 100 eggs in 2 ml of tapwater in the wells of 24 well plates for 48 hours at 24^{o}C and then counting the eggs and L_1 in each replicate. Eggs were examined using both conventional light microscopy and by differential interface contrast microscopy and photographed and compared with those shed by a contemporary MHco3 (ISE) donor lamb.

DNA lysates were prepared from 20 eggs that did not hatch after incubation at 24^{o}C for 48 hours and were judged to have arrested development at a pre-gastrulation stage. PCRs were performed using primers flanking a panel of 5 polymorphic microsatellite markers (3561, X182, X256, Hcms25 and Hcms36). Capillary sequencing in conjunction with Genescan software was used to generate chromatograms for each microsatellite locus and the chromatograms were analysed using GeneMapper software. Observed heterozygosities (H_o), and unbiased estimates of expected heterozygosity (H_e) were calculated using Arlequin version 3.11 software (Nei, 1978; Excoffier and others, 2005).

The MHco3.N1 F_4(2) donor lamb was euthanased 39 days after infection with L_3, following which adult *H. contortus* were picked from the abomasal contents. Next, 140 individual females were picked into PBS and then transferred individually into 1 ml of warm RPMI tissue culture medium in the wells of 24 well plates and incubated in 5% CO_2 at 37^{o}C for 2 hours to promote egg shedding. The adult females were then removed from the 24 well plates containing their egg broods, which were then transferred to a 24^{o}C incubator to permit development and hatching of any fertilised eggs. After 48 hours a drop of helminthological iodine was added to each well to enable eggs and L_1 to be counted. Remaining adult *H. contortus* were stored in absolute ethanol for future study as required.

4.2.1.5 In vitro *bioassays*

EHAs were performed periodically using MHco3.N1 F_3 (4 assays), MHco3.N1 F_4 (2 assays), MHco3.N1 F_4(2) (2 assays), MHco3.N2 F_2 (2 assays) and MHco3.N2 F_3 (3 assays) eggs. Reliable LFIAs were performed periodically using MHco3.N1 F_3 (once), MHco3.N1 F_4 (2 assays), MHco3.N2 F_2 (2 assays) and MHco3.N2 F_3 (once) L_1.

4.2.2 Molecular genetic analysis to assess success of inbreeding

Molecular evidence of successful inbreeding was first sought by examining the filial generations for a reduction in genetic diversity relative to the parental population. Further evidence was sought by assessing whether or not the genotypes of individual filial nematodes were consistent with those expected from a mating between the single female parent of known genotype and a single male parent of unknown genotype. Observed heterozygosities of the inbred filial generations were compared with expected heterozygosity values to enable calculation of an inbreeding coefficient, and pairwise Fst calculations were performed to estimate genetic differences and similarities between populations using Arlequin version 3.11 software (Excoffier and others, 2005).

PCRs were performed using DNA lysate template derived from the parent adult female heads, individual F_1 L_1 and individual F_2, F_3 and F_4 L_3, in addition to the individual heads of MHco3.N1 F_2 and MHco3.N2 F_1 adult *H. contortus*. PCRs were performed on bulk DNA lysate templates prepared from about 500 MHco3.N1 F_2, 500 MHco3.N1 F_3, 500 MHco3.N1 F_4, 500 MHco3.N2 F_2 and 500 MHco3.N2 F_3 L_3.

4.2.2.1 β-tubulin isotype 1 genotypes

PCRs were performed using a primer pair flanking the isotype 1 β tubulin codon 167 and 200 SNPs. PCRs were performed for the N1 and N2 *H. contortus* female parent heads and about 30 individual MHco3.N1 F_1, MHco3.N1 F_2, MHco3.N1 F_3, MHco3.N2 F_1, MHco3.N2 F_2 and MHco3.N2 F_3 *H. contortus*. The F167Y and F200Y genotypes were determined by SNP analysis pyrosequencing. For comparison, the F167Y and F200Y genotypes of 73 and 91 respectively MHco3 (ISE) *H. contortus* were determined using the same method.

4.2.2.2 SSCP genotypes

SSCP genotyping was first performed using three sets of published *H. contortus* primer pairs, in order to define the level of polymorphism in the parental MHco3 (ISE) strain of

H. contortus, and to determine the optimal PCR conditions. PCRs were performed on 4 DNA lysates prepared from individual MHco3 (ISE) adult *H. contortus* heads, using primer pairs flanking polymorphic sections of GluCl α subunit (Blackhall and others, 1998b), GluCl β subunit (Blackhall and others, 1998b) and GABA Cl subunit HG1 (Blackhall and others, 2003) genes (primer sequences and PCR conditions are described in Chapter 2).

GABA Cl SSCP genotyping was then completed on lysates that had been prepared from a single MHco3 (ISE) female *H. contortus* head and a brood of 13 individual L_3 that had hatched to L_1 in RPMI in the well of a 24 well plate and then cultured to L_3 in the same well on *E. coli* and filter paper. This step was performed in order to ensure that maternal haplotypes would be identified in the progeny.

PCRs were performed using a primer pair flanking a region of the GABA Cl subunit HG1 gene for the N1 *H. contortus* female parent's head, 68 individual MHco3.N1 F_1 L_1s, and 80 individual MHco3.N1 F_2 *H. contortus* L_3s. The SSCP genotypes were determined by photographic image analysis of electrophoretically separated, heat and formamide denatured, single strands of PCR product DNA on a non-denaturing polyacrylamide gel. For comparison, PCRs were performed using lysates prepared from 84 MHco3 (ISE) *H. contortus* L_3s and the GABA Cl subunit HG1 SSCP genotypes were determined using the same method.

GABA Cl subunit HG1 PCR products produced from two individual MHco3.N1 F_2 L_3 DNA lysates that were shown to have heterozygous SSCP genotypes (6F and 9G) were run on a 1% agarose electrophoretic gel to separate and enable excision of 305 bp nucleic acid-containing bands. DNA was then isolated from the agarose gel, ligated into pGEM®-T plasmid vectors and transformed into competent cells. Plasmid DNA from the cultured transformed cells was then purified and sequenced in order to confirm a genomic basis for different SSCP haplotypes.

4.2.2.3 Microsatellite genotypes

PCRs were performed on bulk lysates of about 500 MHco3 (ISE), MHco3.N1 F_2, MHco3.N1 F_3, MHco3.N1 F_4, MHco3.N2 F_2 and MHco3.N2 F_3 *H. contortus* using primer pairs flanking a panel of 17 microsatellite loci (8a20, 22co3, 3561, 18210, 26981, 181881, X142, X146, X151, X182, X256, X337, Hcms25, Hcms27, Hcms33, Hcms36 and Hcms40). Capillary sequencing in conjunction with Genescan software was then used to generate chromatograms for each microsatellite locus and the chromatograms were analysed using GeneMapper software. Allele frequencies interpreted from the Genescan traces produced from bulk lysates of the inbred populations were compared with the bulk Genescan traces produced for MHco3 (ISE) *H. contortus*.

PCRs were performed for the N1 and N2 *H. contortus* adult female parent heads and varying numbers of individual MHco3 (ISE), MHco3.N1 F_1, MHco3.N1 F_2, MHco3.N1 F_3, MHco3.N2 F_1, MHco3.N2 F_2 and MHco3.N2 F_3 *H. contortus* using primer pairs flanking a panel of 7 microsatellite loci (8a20, Hcms36, 3561, X182, X256, Hcms25 and 40506) that had been shown to be polymorphic in the bulk Genescan traces produced for the inbred *H. contortus*. Capillary sequencing in conjunction with Genescan software was then used to generate chromatograms for each microsatellite locus and the chromatograms were analysed using GeneMapper software.

4.2.2.3.1 Statistical analysis

Allelic data deduced from the chromatograms was entered onto a Microsoft Excel spreadsheet. Multilocus genotype principal coordinates analysis was conducted using GenAlEx version 6.1 add in software (Peakall and Smouse, 2001) for Microsoft Excel to provide a schematic indication of the degree of inbreeding.

The average number of alleles per locus, observed heterozygosities (H_o), and unbiased estimates of expected heterozygosity (H_e) were calculated using Arlequin version 3.11 software (Nei, 1978; Excoffier and others, 2005). Data were defined as 'standard' rather than 'microsatellite' because the loci did not adhere to the stepwise mutation model

(Redman, *In Press*). Exact tests for Hardy-Weinberg equilibrium were tested per locus using Fisher's exact probability test based on contingency tables (Raymond and Rousset, 1995), where P-values <0.05 were taken as evidence of significant deviation. Probabilities of the Exact significance levels were estimated using 100,000 Markov chain steps. Pairwise linkage disequilibrium was tested for using a likelihood-ratio test (Slatkin and Excoffier, 1996). For each locus, estimates of inbreeding (F_{is}) were calculated using an algorithm based on the formula $(H_e - H_o)/ H_e$. Pairwise Fst values were calculated using Arlequin version 3.11 software. Analysis of Molecular Variance (AMOVA) was performed to test for population differentiation of samples at various levels, locus by locus using the Arlequin version 3.11 software (Excoffier and others, 2005).

4.3 Results

4.3.1 General inbreeding parasitology

4.3.1.1 Surgical transfer of a single male and multiple female day 14 H. contortus *and recovery of progeny of single parent matings*

The three recipient lambs into which a single male and 32, 20, and 20 female day 14 *H. contortus* had been transplanted developed positive FWECs from day 18, 4 days after surgical transfer, reaching about 20 epg by day 21. Male *H. contortus* were not recovered from any of the three recipient lambs, and 12/32, 6/20 and 12/20 (n/transplanted) female *H. contortus* were recovered from the abomasa of recipient lambs A, B and C, respectively (Fig 4.1).

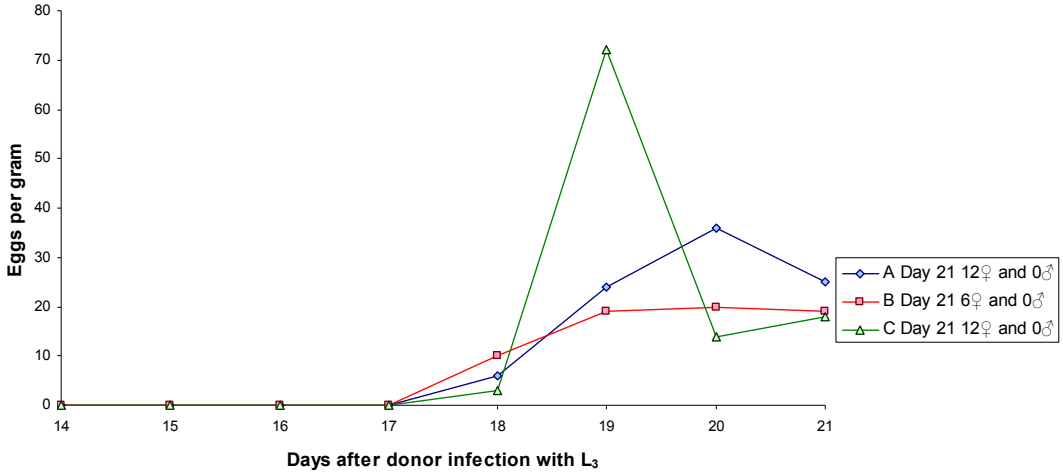


Fig 4.1: FWECs of the three recipient lambs and numbers of adult *H. contortus* recovered from their abomasa, 7 days after surgical transfer.

All of the female *H. contortus* recovered from the 3 recipient lambs shed several hundred eggs during their 8 hour incubation at 37°C in 5% CO_2. About 100 of the eggs shed by each of only 4 *H. contortus* (N1 recovered from recipient lamb A and N2, N3 and N4 recovered from recipient lamb B) developed and hatched to L_1. About 15, 20, 3 and 0 of about 50 L_1 that were cultured from N1, N2, N3 and N4 were recovered from the coprocultures as L_3. Neither L_1 nor L_3 were precisely counted, to avoid possible losses through unnecessary handling.

The *H. contortus* identity of F_1 L_3 cultured from eggs shed by N1, N2, N3 and N4 was confirmed by the identification of appropriate genus or species specific products of ITS2 and NTS PCRs (for example, Fig 4.2).

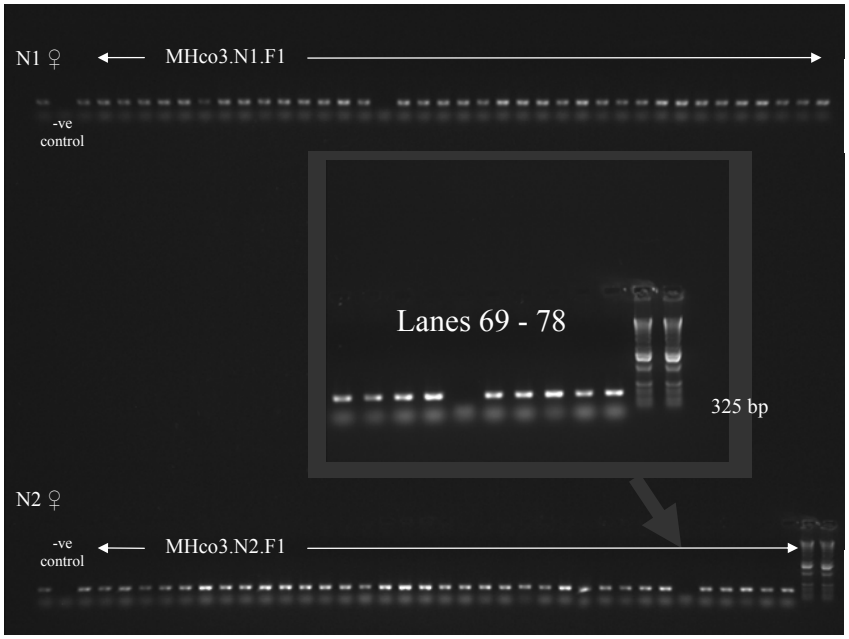


Fig 4.2: ITS2 PCR product run on a 1% agarose gel to show the *Haemonchus* genus identity of N1 (lane 1), MHco3.N1 F_1 (lanes 3 – 40), N2 (lane 41) and MHco3.N2 F_1 L_1 (lanes 43 – 78). Lanes 2 and 42 are negative lysis buffer controls and lanes 79 and 80 are a DNA molecular weight marker X [Roche].

4.3.1.2 Parasitology of filial generations

The MHco3.N1 F_2 donor lamb, that was infected with about 15 F_1 L_3 in order to propagate a filial line had a mean FWEC (± SD) of 1.2 (±1.0) epg between days 20 to 60 after infection with L_3, while the MHco3.N2 F_2 donor lamb, that was infected with about 20 F_1 L_3 had a mean daily FWEC of 22 (±16) epg between days 21 and 64 after infection with L_3. The FWECs of the MHco3.N1 F_3, MHco3.N2 F_3 and MHco3.N1 F_4 donor lambs are shown in Fig 4.3. The mean (± SD) FWEC of the MHco3.N1 F_4 donor lamb that was infected with about 7,500 L_3 was 33 (±29) epg, while that of the MHco3.N1 F_4(2) donor lamb infected with the same number of L_3 was 2898 (± 2491). The shells of the trichostrongyle eggs voided by the filial donor lambs all fluoresced following staining with fluorescein isothiocyanate-labeled peanut agglutinin. The *H. contortus* identity of L_3 and the efficiency of DNA lysate production from individual nematodes were confirmed by larval morphology and by the identification of appropriate products of ITS2 and NTS PCRs (for example, Fig 4.4).

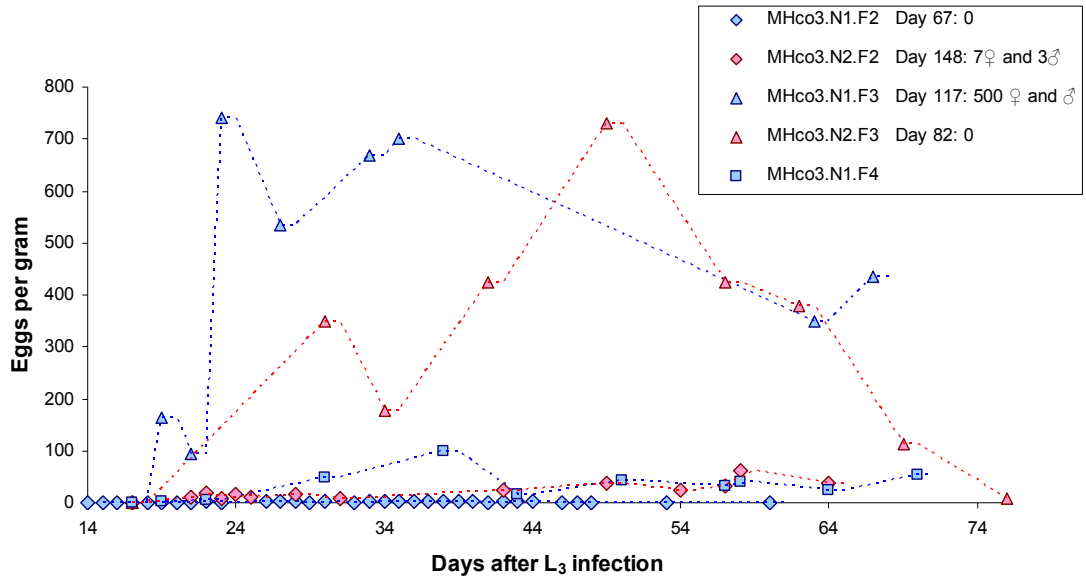


Fig 4.3: FWECs of MHco3.N1 and MHco3.N2 *H. contortus* filial generation donor lambs and numbers of adult *H. contortus* recovered postmortem from the abomasa.

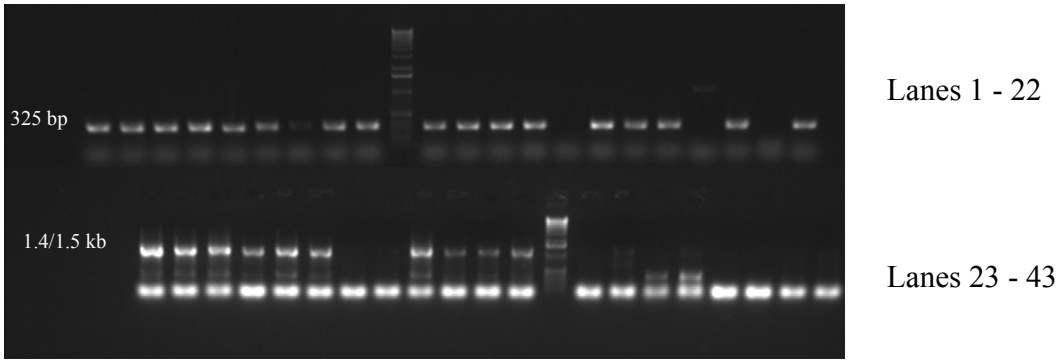


Fig 4.4: Examples of ITS2 (above) and NTS (below) PCR products run on 1% agarose gels to show the *Haemonchus contortus* identity of MHco3.N1 F_3 (lanes 23 – 43, NTS) and MHco3.N2 F_3 L_3 (Lanes 1 – 22 ITS2). DNA has not been amplified from the lysate templates used for lanes 7, 15, 19 and 21 (MHco3.N2 F_3 L_3), and lanes 29, 30, 36, 37, 40, 41, 42 and 43 (MHco3.N1 F_3). Lanes 10 and 35 are a DNA molecular weight marker X [Roche].

4.3.1.3 Egg development and hatching in the inbred MHco3.N1 line

The percentage of MHco3.N1 F_2 eggs that developed and hatched could not be accurately determined, because the FWECs of the donor lamb were too low. Only 30% and 70% respectively of MHco3.N1 F_3 and MHco3.N1 F_4 eggs hatched, compared with an average figure of 97% for MHco3 (ISE) *H. contortus* eggs. Egg hatching in the MHco3.N2 line was similar to that of the parent MHco3 (ISE) *H. contortus*.

The mean (± SD) percentage of 1480 MHco3.N1. $F_4(2)$ eggs that hatched was 33% (±5%), compared with 71% hatching of MHco3.N1 F_4 eggs. Differential interface contrast microscopy of 124 MHco3.N1 $F_4(2)$ eggs showed that their development was arrested predominantly as pre-morphogenesis blastulae, while 124 MHco3 (ISE) *H. contortus* eggs that did not hatch had arrested development predominantly as pre-hatch larvated embryos (Table 4.2) (Figs 5.5).

	MHco3.N1.$F_4(2)$	**MHco3 (ISE)**
Degenerate oocytes	5.7%	6.5%
Two cell arrest	1.6%	0.8%
Pre morphogenesis arrest	89.5%	16.1%
Arrest or abnormalities as pre hatch larvae	3.2%	76.6%

Table 4.2: Stages of developmental arrest in unhatched MHco3.N1 $F_4(2)$ (n=124) and MHco3 (ISE) *H. contortus* eggs.

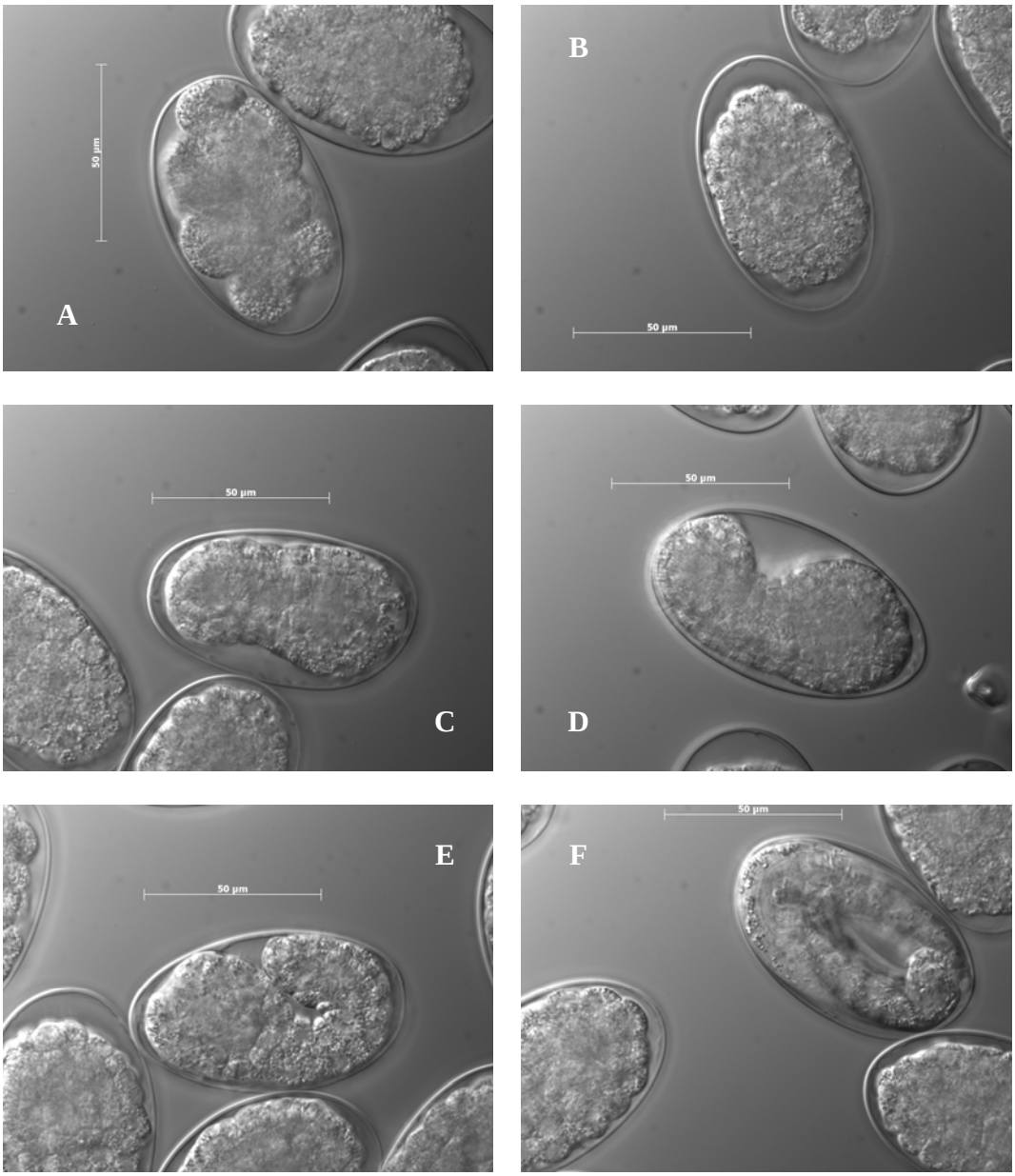


Fig 4.5a: Stages of developmental arrest in MHco3 (ISE) *H. contortus* eggs that did not hatch following 24^{o}C incubation for 48 hours. A: A morula of about 8 blastomeres. B: a pre-morphogenesis about 100 cell blastula. C: Early cell migration to form a gastrula. D and E: early and late 'comma' stages. F: A pre-hatch larva. 76.6% of eggs arrested development as D, E or F.

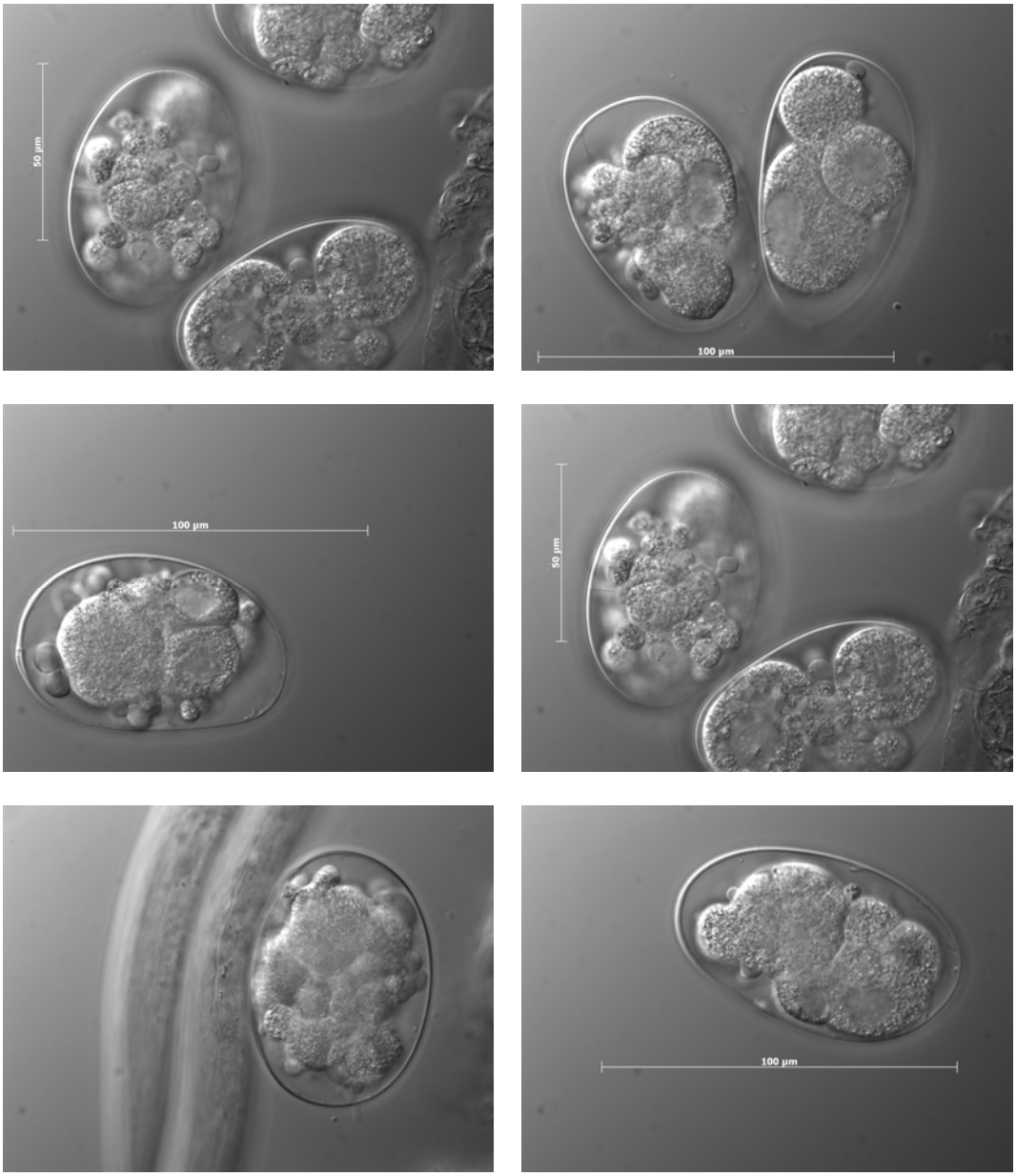


Fig 4.5c: Examples of pre-morphogenesis developmental arrest of MHco3.N1 $F_4(2)$ *H. contortus* morulae. Degenerative changes such as cytoplasmic vaculoation and 'blebbing' of cytoplasmic droplets are visible in both freshly voided eggs and after incubation for 48 hours.

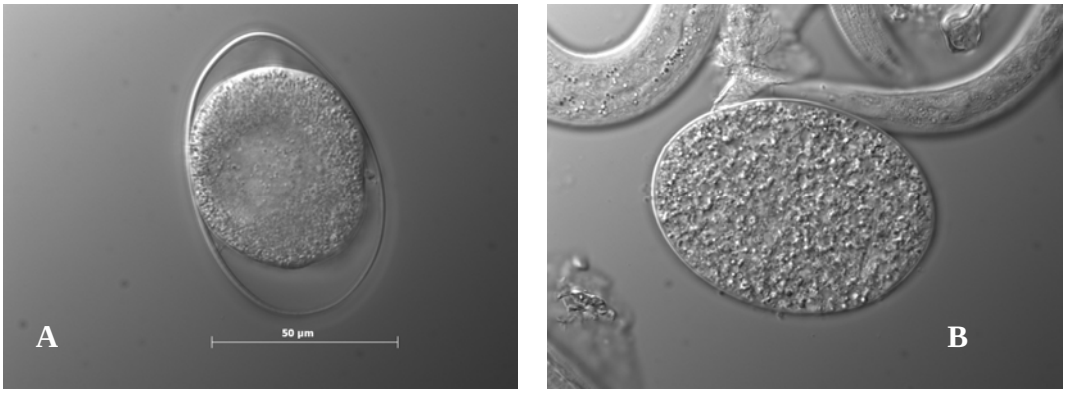


Fig 4.5b: Examples of developmental arrest in MHco3.N1 $F_4(2)$. A: *H. contortus* single cell ova in freshly voided in faeces. B: Bacterial degenerative changes in an arrested embryo 48 hours after extraction from faeces.

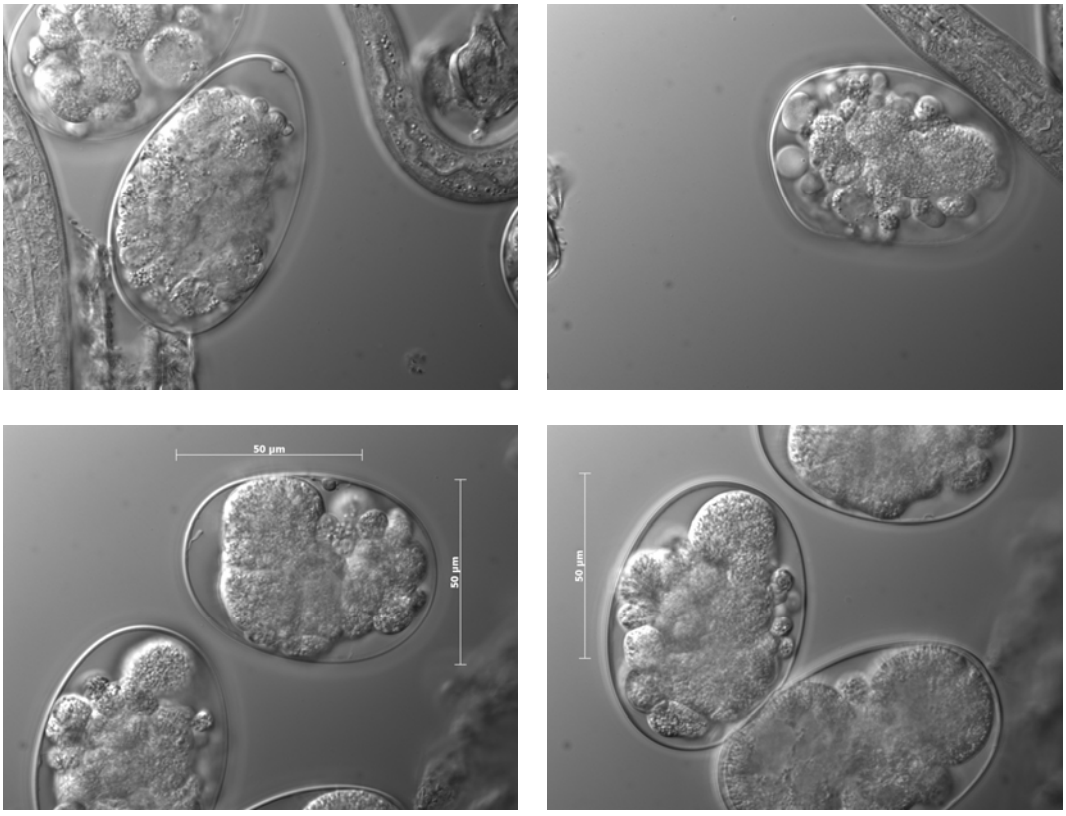


Fig 4.5d: Examples of developmental arrest and degeneration of MHco3.N1 $F_4(2)$ *H. contortus* embryos with some degree of apparent differentiation and perhaps morphogenesis.

Fig 4.5e: Examples of developmental arrest of MHco3.N1 $F_4(2)$ *H. contortus* eggs at various stages of morphogenesis. A and B: arrest at late comma and one-fold stages respectively. C: one-fold arrest with abnormal elongation. D and E: three fold larvae arrested with abnormal morphology and F: a 'normal' looking L_1 in the process of hatching for comparison.

In order to accurately quantify the fecundity and viability of the MHco3.N1 line, the eggs shed by 140 adult female MHco3.N1 F_3 of the MHco3.N1 F_4(2) donor lamb were counted and their percentage hatch assessed. The mean (± SD) number of eggs shed per adult female MHco3.N1 F_3 within 2 hours of postmortem removal from the abomasum was 265 (± 151). The mean (± SD) percentage of these eggs that hatched was 10 (± 10). The number of eggs shed by each individual female nematode was plotted against their percentage to hatch (Fig 4.6) and there was no apparent correlation between the two parameters. Unhatched eggs were mostly thin shelled, but fluoresced weakly following staining with fluorescein isothiocyanate-labeled peanut agglutinin.

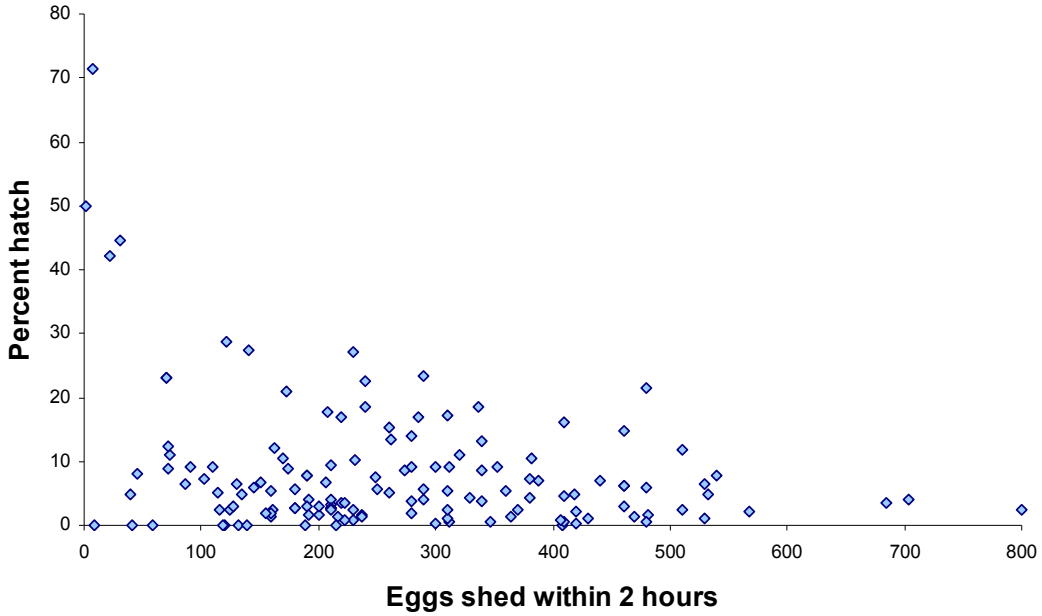


Fig 4.6: Relationship between the numbers of eggs shed by individual female *H. contortus* within 2 hours of postmortem removal from the abomasum of the MHco3.N1 F_4(2) donor lamb and the percentage of eggs hatching.

DNA fingerprinting was performed on 20 eggs that did not hatch after 48 hours incubation using a panel of just 5 polymorphic microsatellite markers. This step was taken in order to determine the presence of heterozygotes, thereby giving a preliminary indication of whether or not the eggs had been fertilised. Five of 20 eggs were

heterozygous at one or more of the panel of five microsatellite loci, including one egg that was heterozygous at 3 loci and 2 eggs that were heterozygous at 2 loci. The data are shown in appendix 4.1. The observed heterozygosities and expected heterozygosities calculated using Arlequin version 3.11 software are shown in Table 4.3.

	3561	**X182**	**X256**	**Hcms25**	**Hcms36**
Number of individuals	20	20	20	19	13
Expected heterozygosity	0.501	0.481	0.501	0.224	0.369
Observed heterozygosity	0.05	0.15	0.15	0.052	0
p-value	<0.001	0.003	0.003	0.011	0.001
Number of alleles	2	2	2	2	2

Table 4.3: Observed and expected heterozygosities of 20 MHco3.N1 F_4(2) eggs that had arrested development at a pre-gastrulation stage, showing heterozygote deficiencies at each locus.

4.3.1.4 In vitro bioassays

In vitro bioassays were performed whenever donor FWECs were high enough and L_1 hatched from eggs shed by contemporary reference *H. contortus* strain donors was satisfactory (refer to chapter 3), in order to characterise the inbred lines. The dose response curves and ED_{50} values for the EHAs are shown in Fig 4.7, alongside those of MHco3 (ISE) *H. contortus* eggs collected from a contemporary donor lamb. The results show low rates of egg hatching in drug free controls for the MHco3.N1 F_3 and MHco3.N1 F_4 *H. contortus*, but that the dose response curves produced from the filial generations of inbred MHco3 *H. contortus* are similar to those for the parental MHco3 (ISE) strain.

	ED_{50} (± SD) (µg/ml thiabendazole)
MHco3 (ISE)	0.044 (±0.007)
MHco3.N1 F_3	0
MHco3.N1 F_4	0.03
MHco3.N2 F_2	0.071 (±0.074)
MHco3.N2 F_3	0.037

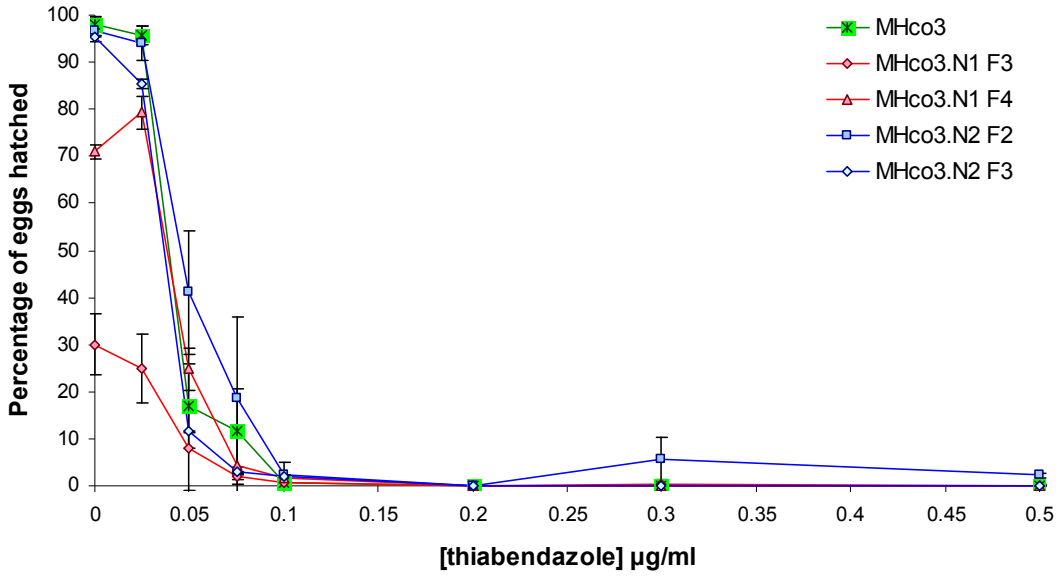


Fig 4.7: EHA dose response curves and ED_{50} values for MHco3 (ISE) *H. contortus* and filial generations of inbred MHco3 *H. contortus*. (Error bars show standard deviations.)

The dose response curves and LFI_{90} and LFI_{99} values for the LFIAs are shown in Fig 4.8, alongside those of MHco3 (ISE) *H. contortus* L_1 hatched from eggs collected from a contemporary donor lamb. The results show that the dose response curves produced from the filial generations of inbred MHco3 *H. contortus* are similar to those for the parental MHco3 (ISE) strain. The percentages of inbred MHco3 *H. contortus* L_1 feeding in drug free control replicates were lower than those of the MHco3 (ISE) *H. contortus*.

	LFI_{90} (± SD) (µg/ml ivermectin)	LFI_{99} (± SD) (µg/ml ivermectin)
MHco3 (ISE)	0.0009 (±0.0004)	0.0014 (±0.0005)
MHco3.N1 F_3	0.001	0.0019
MHco3.N1 F_4	0.0014 (±0.0014)	0.0027 (±0.0022)
MHco3.N2 F_2	0.0002 (±0.0001)	0.0004 (±0.0002)
MHco3.N2 F_3	0.0006	0.0015

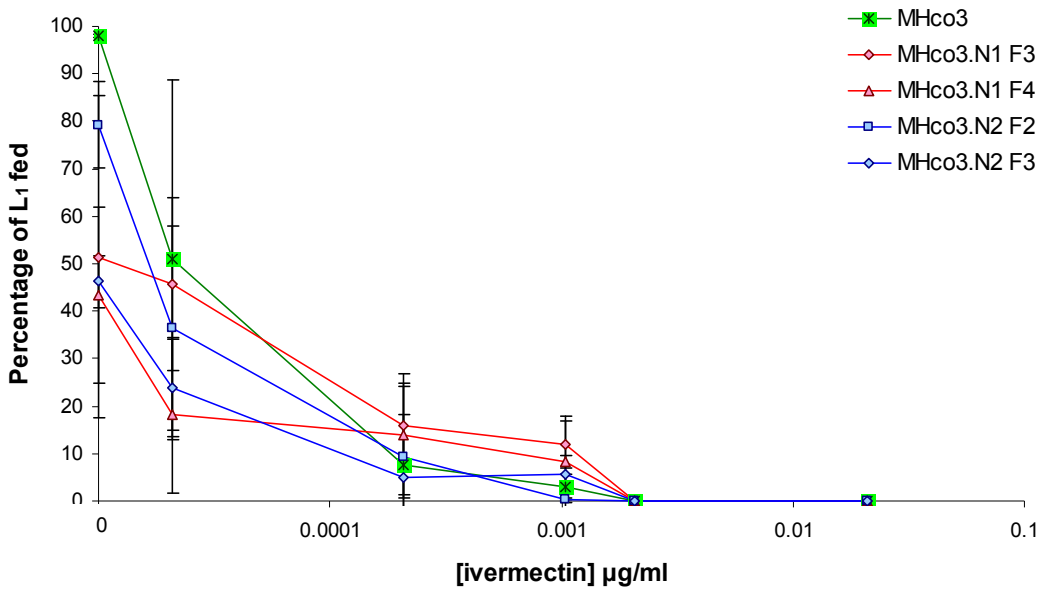


Fig 4.8: LFIA dose response curves and discriminatory drug dose values for MHco3 (ISE) *H. contortus* and filial generations of inbred MHco3 *H. contortus*. (Error bars show standard deviations.)

4.3.2 Molecular characterisation of inbred MHco3.N1 and MHco3.N2 inbred lines

Molecular characterisation of the N1 and N2 female parents and their filial generations was undertaken in order to show a reduction in genetic diversity in the inbred lines and to show that the genotypes of individual filial nematodes were consistent with those expected from a mating between the single female parent of known genotype and a single male parent of unknown genotype.

4.3.2.1 β-tubulin isotype 1 genotypes

The N1 and N2 *H. contortus* female parents that founded the MHco3.N1 and MHco3.N2 lines were both homozygous (*T/T*) at the β-tubulin isotype 1 F200Y and F167Y SNP loci. All of the inbred filial generation nematodes were also homozygous (*T/T*) at both loci. Table 4.4 shows the F200Y and F176Y SNP allele frequencies of the inbred filial generations and of MHco3 (ISE) *H. contortus* (genotype frequencies are also shown in Fig 4.39).

	F200Y			**F167Y**		
	n	***A***	***T***	n	***A***	***T***
MHco3 (ISE)	91	0.05	0.95	73	0.03	0.97
MHco3.N1 F_1	17		1			
MHco3.N1 F_2	31		1	28		1
MHco3.N1 F_3	22		1	18		1
MHco3.N2 F_1	20		1	9		1
MHco3.N2 F_2	24		1	20		1
MHco3.N2 F_3	18		1	17		1

n: number of genotypes

Table 4.4: β-tubulin isotype 1 F200Y and F167Y SNP allele frequencies for MHco3 (ISE) *H. contortus* and the N1 and N2 *H. contortus* inbred filial generations.

4.3.2.2 SSCP genotypes

SSCP genotyping was first performed on DNA lysates prepared from four MHco3 (ISE) *H. contortus* heads to determine whether or not the three published sets of primer pairs would show useful genetic polymorphism. Each MHco3 (ISE) *H. contortus* had a different GABA Cl SSCP genotype, while the GluCl β subunit was less polymorphic and the GluCl α subunit was monomorphic in the same four nematodes (Fig 4.9). Consequently, the GABA Cl subunit HG1 fragment (Blackhall and others, 2003) was chosen as a marker with sufficiently high levels of polymorphism in the parental MHco3 (ISE) strain to provide an initial assessment as to whether there was a significant reduction in polymorphism in the MHco3.N1 and MHco3.N2 inbred lines.

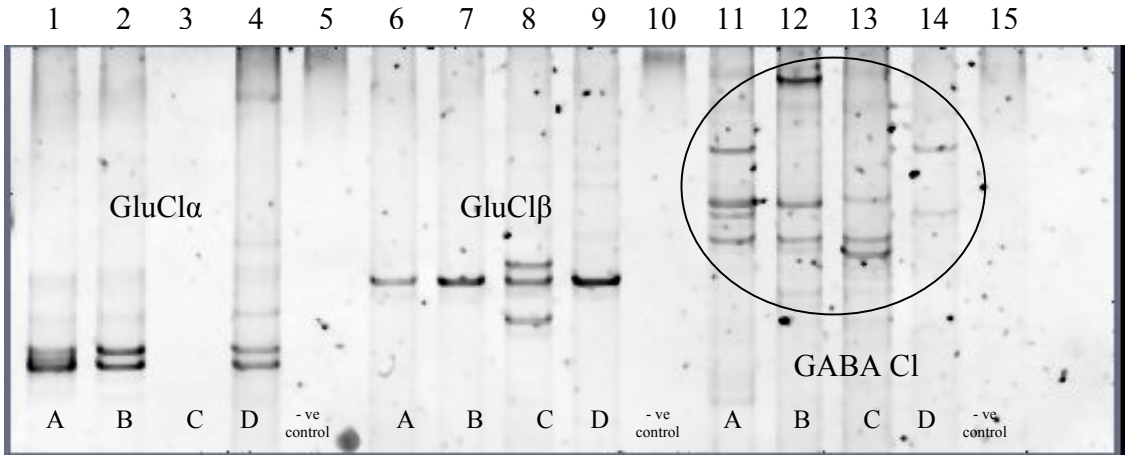


Fig 4.9: SSCP polyacrylamide gel comparing polymorphisms in GluClα (lanes 1 – 4), GluClβ (lanes 6 – 9) and GABA Cl (lanes 11 – 14) subunits of four individual adult MHco3 (ISE) *H. contortus* (1.5 mM $MgSO_4$ and 5 µl of PCR product run on gel). Each of the MHco3 (ISE) *H. contortus* has a different GABA Cl SSCP genotype (circled).

In order to further assess the level of polymorphism of the GABA Cl marker and to ensure that maternal genotypes could be identified in their progeny, a MHco3 (ISE) female *H. contortus* and her brood of 13 individual L_3 were genotyped. SSCP showed the presence of at least 5 different GABA Cl subunit HG1 genotypes in 13 L_3 progeny of a single MHco3 (ISE) female *H. contortus* (Fig 4.10). This provided further evidence of a high level of polymorphism of the GABA Cl subunit HG1 gene in the MHco3 (ISE) strain *H. contortus*. The SSCP profiles are complex and their decoding into separate alleles is not definitive, but it appears broadly that the patterns are consistent with at least one of the alleles present in the female parent being visible in each of the L_3s. The fact that there are at least five different genotypes in the L_3 progeny of a single brood must mean that there is a contribution of more than two paternal genotypes, reflecting polyandrous mating as previously reported.

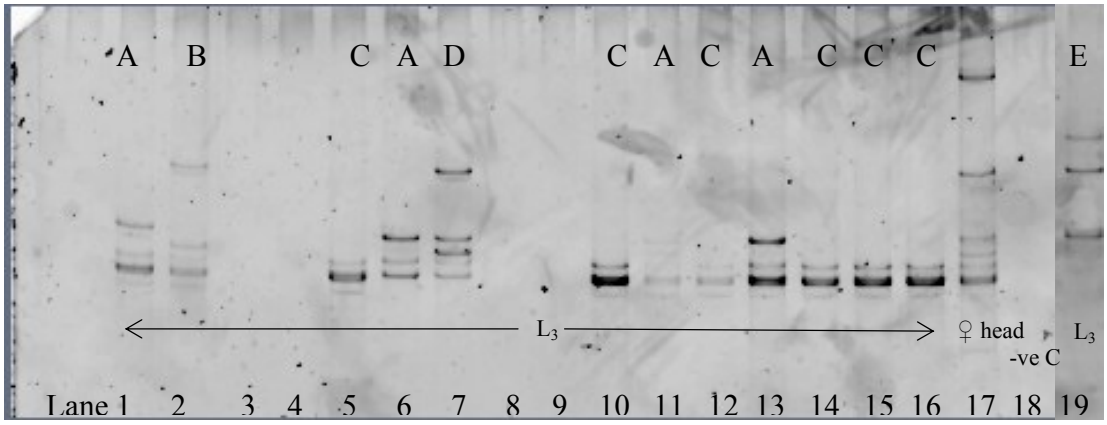


Fig 4.10: A polyacrylamide gel showing the GABA Cl SSCP genotypes of a MHco3 female head (lane 17) and L_3 progeny (lanes 1, 2, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16 and 19) from eggs hatched to L_1 in RPMI in the well of a 24 well plate, and then grown to L_3 in the same well on *E. coli* and filter paper. Amplified PCR product was not seen on an agarose gel corresponding with lanes 3, 4, 8 and 9, while lane 18 is a negative control. Different SSCP genotypes are labeled A – E.

Eighty-four parental strain MHco3 (ISE) *H. contortus* L_3 were genotyped in order to further demonstrate the level of polymorphism in the GABA Cl subunit HG1 marker. Although the complexity of the SSCP profiles make it difficult to unequivocally identify individual alleles, the individual SSCP profiles give an indication of the overall level of polymorphism of the MHco3(ISE) strain at this locus. The SSCP showed the presence of at least 15 different GABA Cl subunit HG1 genotypes in MHco3 (ISE) *H. contortus*. Examples of SSCP profiles from 55 individual MHco3 (ISE) *H. contortus* are shown in Fig 4.11.

Fifty-seven F_1 and 65 F_2 progeny of the MHco3.N1 inbred line were genotyped and only 3 different SSCP profiles were identified (examples are shown in Figs 5.12 and 5.13). Indeed, because of the reduced level of polymorphism it is possible to decode the profiles into alleles for this inbred line. The founding N1 female parent (lanes 5, 15, 25 and 35 of Fig 4.12) has two alleles, one of which is present in all of the F_1 and F_2 progeny.

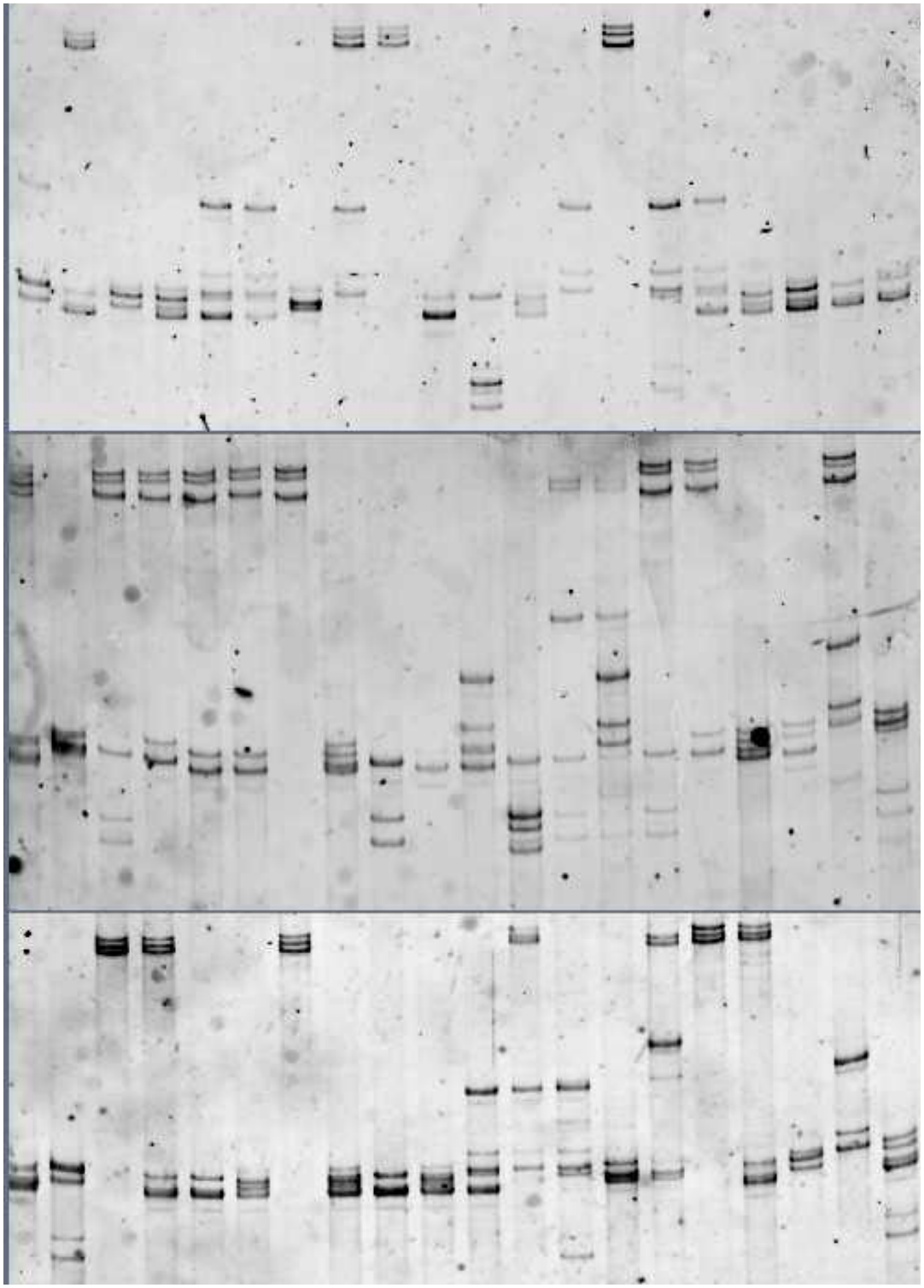


Fig 4.11: SSCP polyacrylamide gels showing a high level of polymorphism in MHco3 (ISE) *H. contortus*. Each lane shows the SSCP GABA Cl subunit HG1 genotype of an individual L_3.

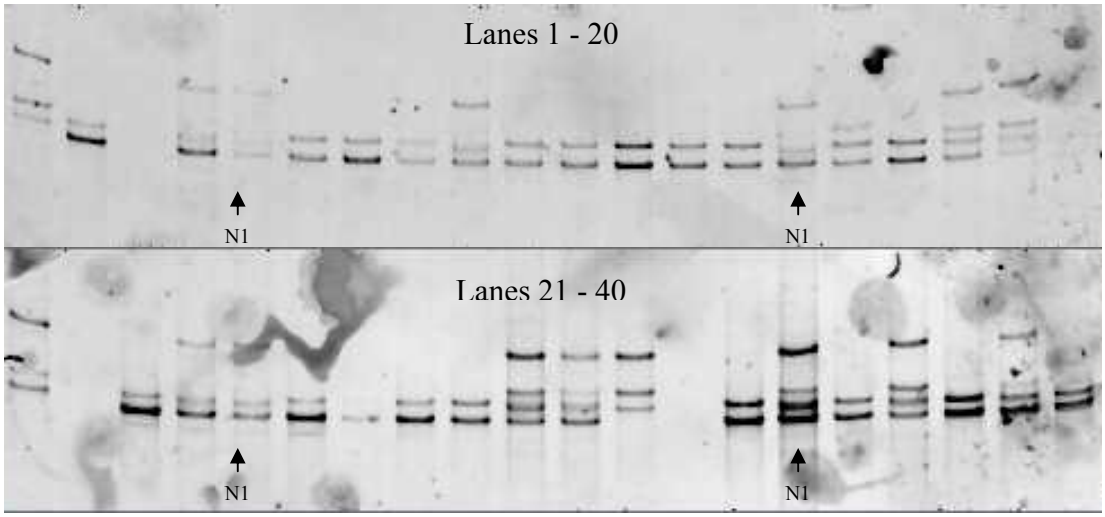


Fig 4.12: Polyacrylamide gels showing the GABA Cl SSCP genotypes of the N1 female (lanes 5, 15, 25 and 35) and of individual MHco3.N1.F_1 L_1s (lanes 1, 2, 4. 6 – 14, 16 – 19, 21, 23, 24, 26 – 32, 34, and 36 – 40).

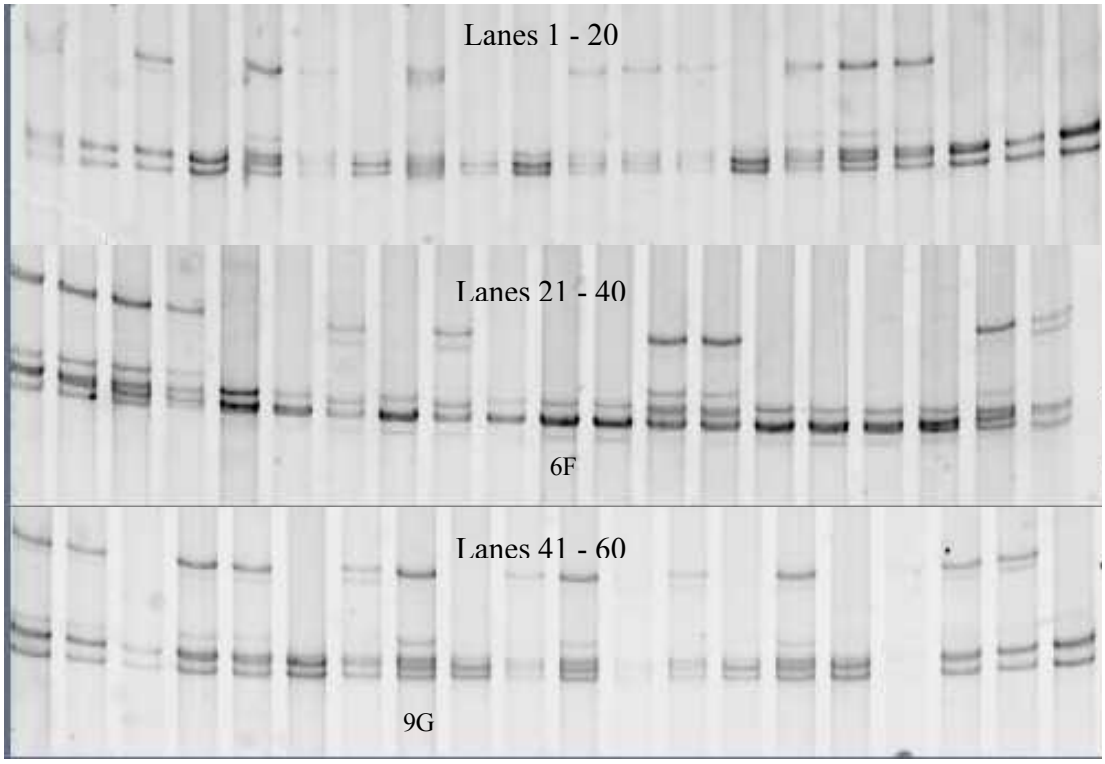


Fig 4.13: Polyacrylamide gels showing the GABA Cl SSCP genotypes of MHco3.N1.F_2 L_3. (Agarose gel PCR product bands for heterozygous 6F (corresponding with lane 31) and 9G (corresponding with lane 48) were excised, ligated, transformed, cloned and sequenced.)

The GABA Cl subunit HG1 region PCR products (Fig 4.14) from two heterozygous MHco3.N1.F_2 worms (6F and 9G in Fig 4.13) were cloned and sequenced (12 clones for 6F and 11 clones for 9G) in order to identify differences in DNA sequences responsible for the different SSCP haplotypes. This information would support the validity of the interpretation of SSCP genotypes.

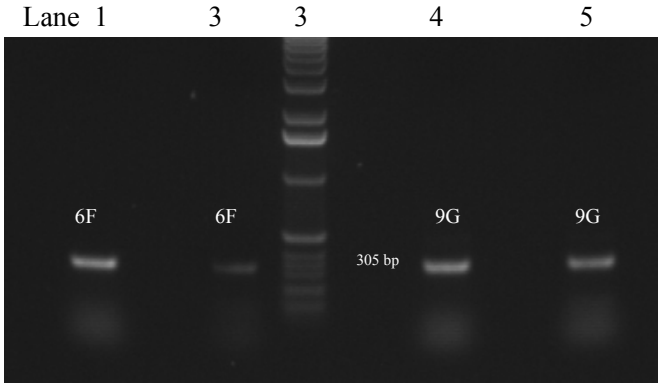
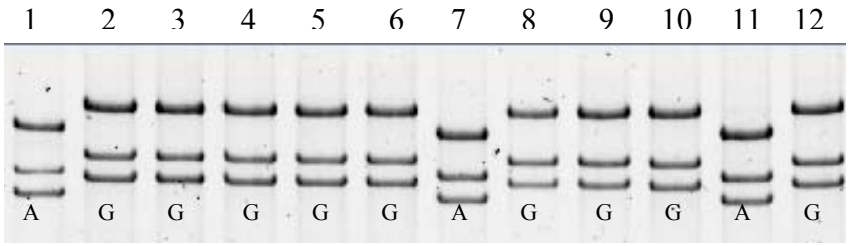


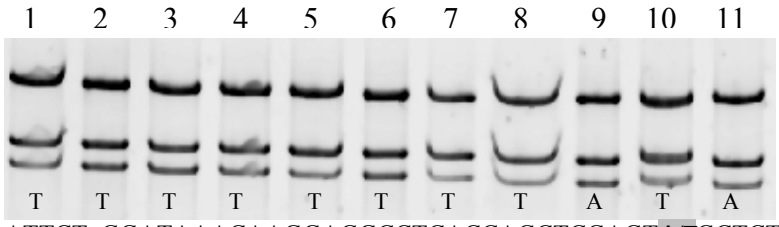
Fig 4.14: GABA Cl PCR product for MHco3.N1.F_2 6F (lanes 1 and 3) and 9G (lanes 4 and 5) prepared for excision of bands from the 1% agarose gel. The product in lanes 1 and 3 and in lanes 4 and 5 were produced from the same PCRs. Lane 3 is a DNA molecular weight marker X [Roche]

SSCP of plasmid DNA cloned from the MHco3.N1.F_2 worms 6F and 9G showed the identity of both haplotypes of each worm (Figs 5.15 and 5.16). The sequencing data were consistent with the different haplotypes being due to SNPs at positions 118 (worm 6F) and 38 (worm 9G) of the sequenced section of GABA Cl subunit HG1 gene.



ATTCT_GGATAAACAAGGAGGCCTCACCAGCTCGAGTTGCTCTAGGTTAAAATTCAGAATAC
AGTAATTAAAACGATTGCAAAAATGAAAATTAGATGTTAGGAATTATGACGGT*G/A*TTGTCAA
TGTCTACTCTAGGCTTTGGGCTGCGCACCGACTTGCCGAAAGTCTCCCATTCGACAGCGCTTG
ACA----------

Fig 4.15: GABA Cl SSCP haplotypes of the MHco3.N1.F_2 6F worm. Each lane shows the haplotype of a separate clone of plasmid DNA. The haplotypes in lanes 1, 7 and 11 correspond with the presence of adenine (A) at position 118 of the sequenced section of the GABA Cl subunit HG1 gene, while the haplotypes in lanes 2, 3, 4, 5, 6, 8, 9, 10 and 12 correspond with the presence of guanine (G) at position 118. The sequence data shown are consistent with the different haplotypes being due to the presence of a *G/A* SNP.



ATTCT_GGATAAACAAGGAGGCCTCACCAGCTCGAGT*A/T*GCTCTAGGTTAAAATTCAGAATA
CAGTAATTAAAACGATTGCAAAAATGAAAATTAGATGTTAGGAATTATGACGGTGTTGTCAA
TGTCTACTCTAGGCTTTGGGCTGCGCACCGACTTGCCGAAAGTCTCCCATTCGACAGCGCTTG
ACA---------

Fig 4.16: GABA Cl SSCP haplotypes of the MHco3.N1.F_2 9G worm. Each lane shows the haplotype of a separate clone of plasmid DNA. The haplotypes in lanes 9 and 11 correspond with the presence of adenine (A) at position 38 of the sequenced section of the GABA Cl subunit HG1 gene, while the haplotypes in lanes 2, 3, 4, 5, 6, 8, 9, 10 and 12 correspond with the presence of thymine (T) at position 38. The sequence data shown are consistent with the different haplotypes being due to the presence of an *A/T* SNP.

4.3.2.3 Microsatellite genotypes

4.3.2.3.1 Interpretation of bulk lysate Genescan data

The microsatellite Genescan traces for a panel of 17 microsatellites were first examined for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines, in order to demonstrate loss of polymorphism in the inbred lines, consistent with inbreeding. Only microsatellite alleles that were present in the parental MHco3 (ISE) strain were identified in the inbred lines (with the exception of possible Hcms40 alleles in the MHco3.N1 line). Differences were seen at the 8a20, 22co3, 3561, 18210, X142, X256, X142, Hcms25, Hcms33, Hcms36 and Hcms40 loci, involving loss of alleles in the inbred lines compared to the MHco3 (ISE) parental strain. The discernable alleles present in the Genescan traces for the 17 microsatellite loci are shown in Table 4.5. Fig 4.17 provides an example of the Genescan traces for microsatellite locus Hcms33, showing the absence of allele 218 in the MHco3.N2 line which was present in the parental MHco3 (ISE) strain. Genescan traces for all 17 microsatellite loci are shown in appendix 4.2.

4.3.2.3.2 Interpretation of individual nematode Genescan data

The microsatellite genotypes of the N1 and N2 female *H. contortus* parents and of individual inbred MHco3.N1 and MHco3.N2 filial nematodes were determined to enable genetic analysis of inbreeding. Several lysates of individual nematodes failed to amplify in the PCR. While it was not possible to determine whether this was due to poor template quality or to failure of primers to anneal caused by sequence polymorphism (null alleles), the former explanation is most likely. Microsatellite genotypes, including those for failed PCR amplification are shown in appendix 4.3.

Table 4.6 shows the microsatellite allele frequencies based on genotyping individual nematodes of the MHco3 (ISE) *H. contortus*, the N1 and N2 female parents of the inbred lines, and in their MHco3.N1 and MHco3.N2 filial generations. The allele frequencies for X chromosome X182 and X256 microsatellites are based on an assumption that half of the larvae are XO males and only have one allele. (Data are not shown in Table 4.6

for the individual nematodes where PCRs failed to amplify the microsatellite markers, because most cases probably arose due to poor DNA lysate template quality, rather than because they were true null alleles.) Precise analysis of these data was not possible, due to the presence of large numbers of failed PCRs for each microsatellite marker. With the exception of locus 3561, the allele frequencies determined by PCR of the individual nematode lysates are in agreement with those estimated from the subjective interpretation of the bulk lysate Genescan traces. Furthermore, the allele frequencies determined by individual L_3 genotyping of the parental MHco3 (ISE) strain were similar to those previously determined at the Glasgow University Veterinary School (Libby Redman, *data on file*).

Marker	MHco3 (ISE)	N1 female	MHco3.N1 F_2	MHco3.N1 F_3	MHco3.N1 F_4
8a20	192, 196, 232, 236, 240	**196.196**	196	196	196
22co3	234, 250	*	250	250	
3561	261, 285	**261.261**	261	261	261
18210	219, 225	*	219, 225	219, 225	
26981	293	*	293	293	
181881	247	*	247	247	
X142	178, 179, 180	*	179	179	
X146	147, 151	*	147, 151	147, 151	
X151	226, 234	*	226, 234	226, 234	
X182	372, 374	**372.372**	372	372, 374	372, 374
X256	239, 241, 245	**239.239**	239, 241	239, 241	239, 241
X337	331, 339	*		331	
Hcms25	207, 209, 211, 215, 217	**211.217**	211, 217	211, 217	211, 217
Hcms27	358	**358.358**	358	358	
Hcms33	204, 218	*	204, 218	204, 218	
Hcms36	148, 152	**148.152**	148, 152	148, 152	148, 152
Hcms40	285, 297	*	283[1]	281[1]	

* not genotyped 1: unexplained alleles possibly due to PCR or capillary sequencing errors

Marker	MHco3 (ISE)	N2 female	MHco3.N2 F_2	MHco3.N2 F_3
8a20	192, 196, 232, 236, 240	**232.232**	232	192, 196, 232
22co3	234, 250	*	250	250
3561	261, 285	**261.287**	261, 285, 287	261, 285, 287
18210	219, 225	*	219	219
26981	293	*	293	293
181881	247	*	247	247
X142	178, 179, 180	*	178	179
X146	147, 151	*	147, 151	147, 151
X151	226, 234	*	226, 234	226, 234
X182	372, 374	**372.374**	372, 374	372, 374
X256	239, 241, 245	**241.245**	239, 241, 245	239, 241, 245
X337	331, 339	*	331, 339	331, 339
Hcms25	207, 209, 211, 215, 217	**211.217**	209, 211, 215, 217	209, 211, 215, 217
Hcms27	358	**358**	358	358
Hcms33	204, 218	*	204	204
Hcms36	148, 152	**148.148**	148	148
Hcms40	285, 297	*	297	297

* not genotyped

Table 4.5: Discernable alleles that could be amplified from the bulk lysate preparations of MHco3 (ISE) *H. contortus* and the inbred MHco3.N1 and MHco3.N2 inbred lines. Known genotypes of the N1 and N2 founding female parents are shown in the third column. Alleles that are present in the MHco3 (ISE) parental strain, but not in the inbred lines are highlighted.

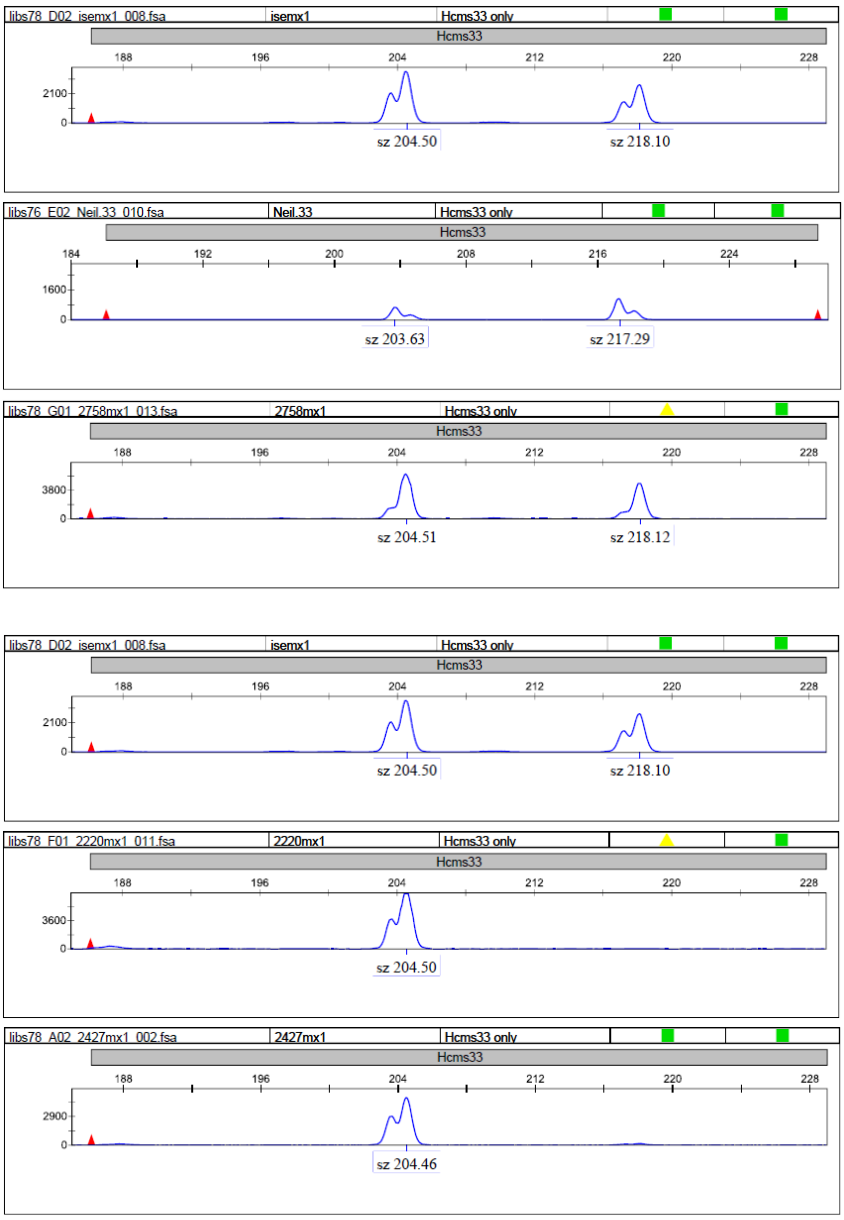


Fig 4.17: Microsatellite locus Hcms33 Genescan traces for bulk DNA lysate preparations of MHco3 (ISE) and inbred MHco3.N1 and MHco3.N2 *H. contortus* filial generations, showing different genotypes in the two inbred lines, with an absence of allele 218 in the MHco3.N2 *H. contortus*.

8a20		**n**	**192**	**196**	**208**	**212**	**214**	**220**	**232**	**240**	**244**	**248**
MHco3 (ISE)	L3	54	30	13					35	22		
MHco3.N1	Adult ♀	2		100								
MHco3.N1.F1	L1	72		96		1	3					
MHco3.N1.F2	L3	54		96	4							
MHco3.N1.F2	Adults	36		100								
MHco3.N1.F3	L3	66		97				1				2
MHco3.N1.F4	L3	32		91		3					3	3
Hcms36		**n**	**148**	**152**								
MHco3 (ISE)	L3	54	63	37								
MHco3.N1	Adult ♀	2	50	50								
MHco3.N1.F1	L1	36	67	33								
MHco3.N1.F2	L3	10	50	50								
MHco3.N1.F2	Adults	30	37	63								
MHco3.N1.F3	L3	12	33	67								
MHco3.N1.F4	L3	24	38	62								
3561		**n**	**261**	**285**	**287**							
MHco3 (ISE)	L3	56	54	41	5							
MHco3.N1	Adult ♀	2	100									
MHco3.N1.F1	L1	86	83	17								
MHco3.N1.F2	L3	24	46	54								
MHco3.N1.F2	Adults	92	66	34								
MHco3.N1.F3	L3	58	76	24								
MHco3.N1.F4	L3	26	92	8								
X 182		**n**	**372**	**374**								
MHco3 (ISE)	L3	49	63	37								
MHco3.N1	Adult ♀	2	50	50								
MHco3.N1.F1	L1	65	82	18								
MHco3.N1.F2	L3	25	68	32								
MHco3.N1.F2	Adults	72	68	32								
MHco3.N1.F3	L3	47	64	36								
MHco3.N1.F4	L3	26	46	54								
X 256		**n**	**239**	**241**	**245**							
MHco3 (ISE)	L3	50	58	36	6							
MHco3.N1	Adult ♀	2	100									
MHco3.N1.F1	L1	64	78	22								
MHco3.N1.F2	L3	33	82	12	6							
MHco3.N1.F2	Adults	84	68	32								
MHco3.N1.F3	L3	28	75	25								
MHco3.N1.F4	L3	38	66	34								
Hcms25		**n**	**209**	**211**	**213**	**215**	**217**					
MHco3 (ISE)	L3	54	6	28	48	6	13					
MHco3.N1	Adult ♀	2		50			50					
MHco3.N1.F1	L1	88		48			52					
MHco3.N1.F2	L3	34		29			71					
MHco3.N1.F2	Adults	88		31			69					
MHco3.N1.F3	L3	56		41			59					
MHco3.N1.F4	L3	36		19			81					
Hcms27		**n**	**338**	**358**								
MHco3 (ISE)	L3	LR	7	93								
MHco3.N1	Adult ♀	2		100								
MHco3.N1.F1	L1	76		100								
MHco3.N1.F2	L3	10		100								
MHco3.N1.F2	Adults	44		100								
MHco3.N1.F3	L3	18		100								
MHco3.N1.F4	L3	4		100								

n: number of alleles determined (for the autosomal markers, this figure is 2 x the number of worms genotyped, but for the X chromosome markers an assumption has been made that half of the larvae are XO male)

LR: data from Libby Redman

Table 4.6 i): Allele frequencies of the N1 founding female parents and the inbred MHco3.N1 filial generations at 7 microsatellite loci, compared with allele frequencies of MHco3 (ISE) L_3 *H. contortus* at the same microsatellite loci.

8a20		**n**	**192**	**196**	**208**	**212**	**214**	**220**	**232**	**240**	**244**	**248**
MHco3 (ISE)	L3	54	30	13					35	22		
MHco3.N2	Adult ♀	2							100			
MHco3.N2.F1	L1	22							100			
MHco3.N2.F1	L3	14	14						86			
MHco3.N2.F2	Adults	64	20	1	2		3	8	66			
MHco3.N2.F3	L3	48	48				4	6	42			

Hcms36		**n**	**148**	**152**
MHco3 (ISE)	L3	54	63	37
MHco3.N2	Adult ♀	2	100	
MHco3.N2.F1	L1	16	100	
MHco3.N2.F1	L3	10	90	10
MHco3.N2.F2	Adults	28	100	
MHco3.N2.F3	L3	22	100	

3561		**n**	**261**	**285**	**287**
MHco3 (ISE)	L3	56	54	41	5
MHco3.N2	Adult ♀	2	50		50
MHco3.N2.F1	L1	26	35	15	50
MHco3.N2.F1	L3	20	25	25	50
MHco3.N2.F2	Adults	64	23	42	34
MHco3.N2.F3	L3	44	23	41	36

X 182		**n**	**372**	**374**
MHco3 (ISE)	L3	49	63	37
MHco3.N2	Adult ♀	2	50	50
MHco3.N2.F1	L1	30	37	63
MHco3.N2.F1	L3	17	24	76
MHco3.N2.F2	Adults	56	45	55
MHco3.N2.F3	L3	43	42	58

X 256		**n**	**239**	**241**	**245**
MHco3 (ISE)	L3	50	58	36	6
MHco3.N2	Adult ♀	2		50	50
MHco3.N2.F1	L1	40	28	30	43
MHco3.N2.F1	L3	18	39	44	17
MHco3.N2.F2	Adults	35	37	29	34
MHco3.N2.F3	L3	37	65	16	19

Hcms25		**n**	**209**	**211**	**213**	**215**	**217**
MHco3 (ISE)	L3	54	6	28	48	6	13
MHco3.N2	Adult ♀	2		50			50
MHco3.N2.F1	L1	36	22	28		6	44
MHco3.N2.F1	L3	20	20	20		20	40
MHco3.N2.F2	Adults	44	25	18		23	34
MHco3.N2.F3	L3	46	9	30		24	37

Hcms27		**n**	**358**	**358**
MHco3 (ISE)	L3	LR	7	93
MHco3.N2	Adult ♀	2		100
MHco3.N2.F1	L1	30		100
MHco3.N2.F1	L3	16		100
MHco3.N2.F2	Adults	42		100
MHco3.N2.F3	L3	30		100

n: number of alleles determined (for the autosomal markers, this figure is 2 x the number of worms genotyped, but for the X chromosome markers an assumption has been made that half of the larvae are XO male)

LR: data from Libby Redman

Table 4.6 ii): Allele frequencies of the N2 founding female parents and the inbred MHco3.N2 filial generations at 7 microsatellite loci, compared with allele frequencies of MHco3 (ISE) L_3 *H. contortus* at the same microsatellite loci.

4.3.2.3.3 Principal coordinates analyses

PCA was performed on multilocus genotypes for a panel of 6 microsatellite markers (8a20, 3561, X182, X256, Hcms25 and Hcms36) in the MHco3 (ISE) *H. contortus* and in the F_1 inbred MHco3.N1 and MHco3.N2 *H. contortus* lines. Fig 4.18 shows the variation in the first three multilocus genotype coordinates. Each point represents an individual nematode, and coordinates are only shown for genotypes with no failed PCR due to poor quality template or homozygous null loci. The principal coordinates for the N1 and N2 founding female parents are shown. The scattering of the data points reflects the level of genetic variation for each population. It can be seen that the data points, representing individual multilocus genotypes, are more scattered for the parental MHco3(ISE) strain than for the MHco3.N1 F_1 and MHco3.N2 F_1 nematodes. It is notable that the clusters of the MHco3.N1 F_1 and MHco3.N2 F_1 multilocus genotypes (small squares) surround the multilocus genotype of their respective founding female parents (large squares). This suggests that the genetic variation has been reduced by the single parent mating procedure. Furthermore, the MHco3.N1 F_1 and MHco3.N2 F_1 data points cluster quite separately, demonstrating that the two F_1 populations are genetically differentiated from each other.

Fig 4.19 shows the variation in the first three multilocus genotype coordinates for a panel of 6 microsatellite markers (8a20, 3561, X182, X256, Hcms25 and Hcms36) in the MHco3 (ISE) *H. contortus* and in the inbred MHco3.N1 and MHco3.N2 lines of *H. contortus*. Each point represents an individual nematode, and coordinates are only shown for genotypes with failed PCRs due to poor quality template at one or less loci (arbitrarily analysed as nulls). The plots show that population sub-structuring between the MHco3 (ISE) *H. contortus* and the two inbred lines is maintained in the inbred filial generations. Increased scatter in the filial populations suggests that their reduced genetic diversity has not been maintained, but may be an artifact caused by incorrect allocation of null alleles. The equivalent PCA plots generated by only using genotypes with no failed PCR due to poor quality template or null loci are shown in Fig 4.20. While the number of multilocus genotypes for the inbred MHco3.N2 line is small, these plots show

clustering of the data points for both inbred lines consistent with inbreeding. Comparison of the PCA plots shown in Fig 19 and in Fig 20 also illustrates some of the data analysis problems caused by poor quality DNA template or null loci. The data used to generate the PCA plots are shown in appendices 4.4 and 4.5.

(Note: The PCA plots are shown in order to provide a graphic description of inbreeding and population structure based on multilocus genotypes. An explanation is provided in section 3.3.2.3 of this thesis to aid in the interpretation of the plots. It is acknowledged that these plots should be accompanied by a components loading matrix in order to demonstrate their significance.)

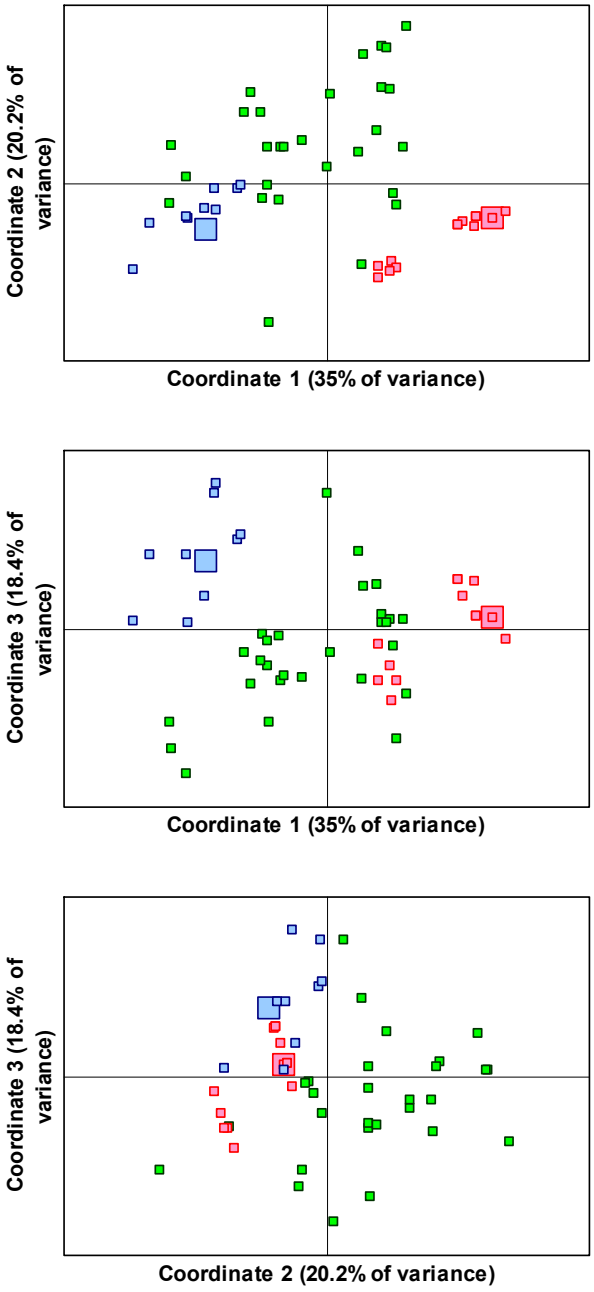


Fig 4.18: PCA plots to show the population genetic structures of MHco3 (ISE) *H. contortus* and the MHco3 N1 F_1 and MHco3.N2 F_1 populations using a panel of 6 microsatellite markers. The principal coordinates for the N1 and N2 female parents are shown. Multilocus genotypes are only included for individual worms with no failed PCR due to poor quality template or null loci.

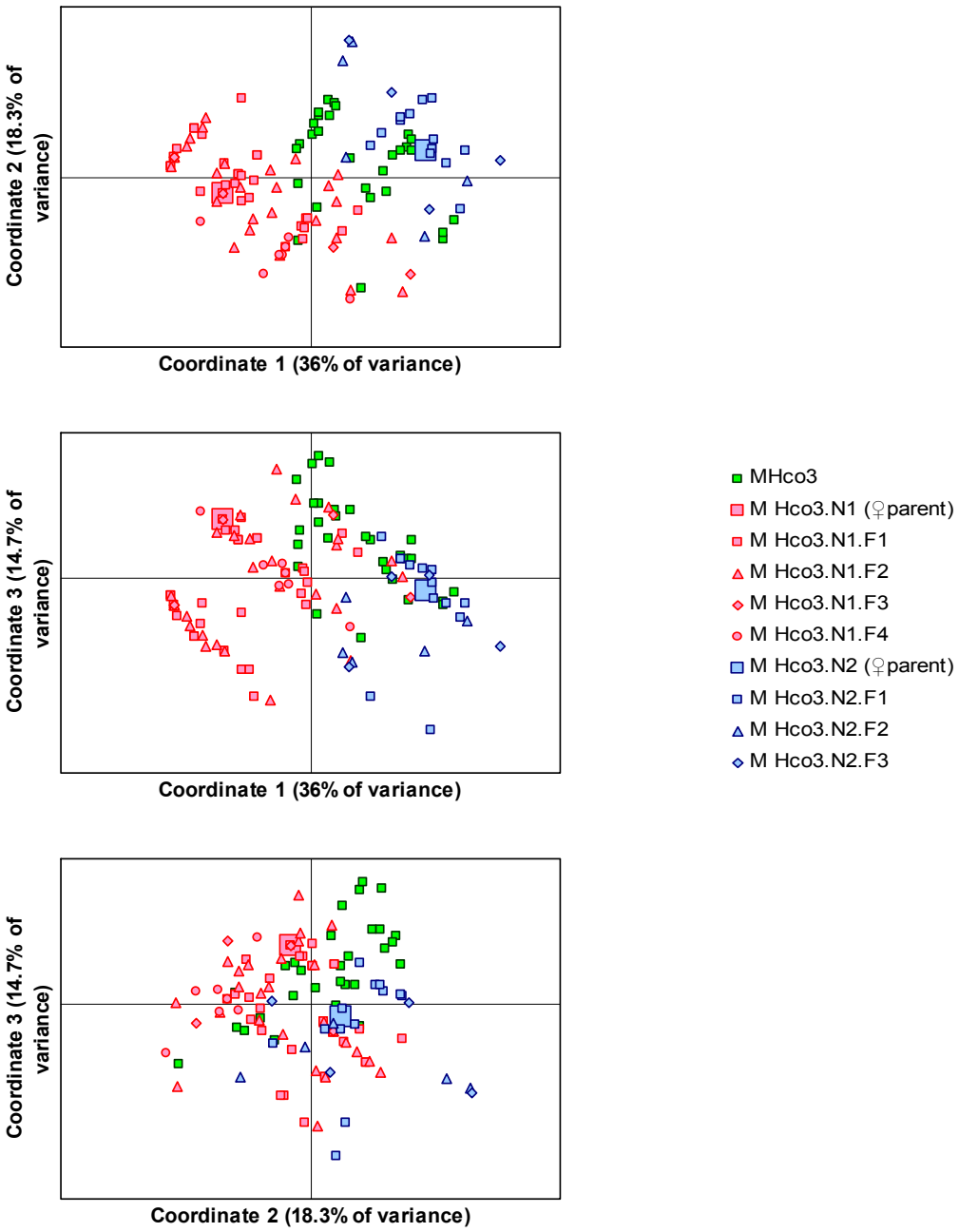


Fig 4.19: PCA plots to show the population genetic structures of MHco3 (ISE) *H. contortus* and the inbred MHco3.N1 and MHco3.N2 lines using a panel of 6 microsatellite markers. The principal coordinates for the N1 and N2 female parents are shown. Multilocus genotypes are included for individual worms with failed PCRs due to poor quality template at one or less loci.

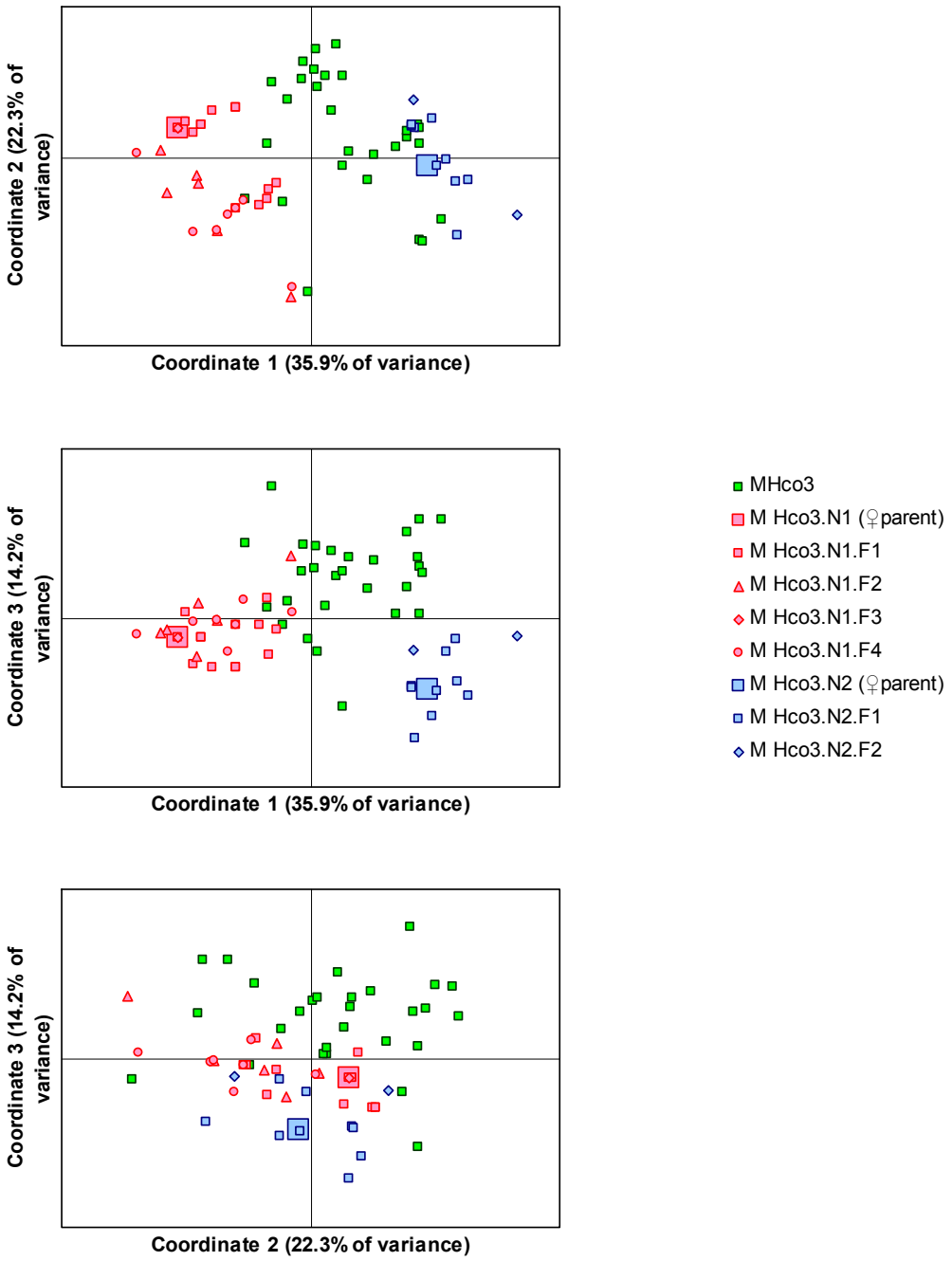


Fig 4.20: PCA plots to show the population genetic structures of MHco3 (ISE) *H. contortus* and the inbred MHco3.N1 and MHco3.N2 lines using a panel of 6 microsatellite markers. The principal coordinates for the N1 and N2 female parents are shown. Multilocus genotypes are only included for individual worms with no failed PCR due to poor quality template or null loci.

4.3.2.3.4 Calculation of inbreeding coefficients

The average number of alleles per locus, observed heterozygosities (H_o), and unbiased estimates of expected heterozygosity (H_e) are shown in Table 4.7. F_{is} values of -1 and +1 provide evidence of no inbreeding and being genetically indistinguishable respectively, and comparison of the indices for the MHco3.N1 and MHco3.N2 lines with MHco3 gives an indication of the level of inbreeding. Overall both F_2 populations appear to be more inbred than the reference MHco3 (ISE) population, from which they were derived.

Population		**8a20**	**Hcms36**	**3561**	**X182**	**X256**	**Hcms25**	**All loci**
MHco3.N1.F_1	N	33	17	35	35	35	36	
	H_e	0.089	0.451	0.325	0.288	0.325	0.501	
	H_o	0.03	0.647	0.4	0.3342	0.343	0.389	
	p-value	0.015	0.109	0.303	0.558	1	0.2	
	F_{is}	**0.663**	**-0.455**	**-0.236**	**-0.193**	**-0.057**	**0.226**	**-0.287**
	A	3	2	2	2	2	2	
MHco3.N1.F_2	N	*	20	52	52	50	45	
	H_e	*	0.492	0.468	0.457	0.453	0.425	
	H_o	*	0.5	0.423	0.192	0.24	0.467	
	p-value	*	1	0.552	<0.001	0.001	0.72	
	F_{is}	*****	**-0.016**	**0.097**	**0.582**	**0.473**	**-0.1**	**0.337**
	A	*	2	2	2	2	2	
MHco3.N2.F_1	N	14	13	19	19	19	16	
	H_e	0.138	0.077	0.622	0.422	0.676	0.732	
	H_o	0	0.077	0.526	0.474	0.737	0.813	
	P-value	0.036	1	0.009	1	0.747	0.738	
	F_{is}	**1**	**0**	**0.157**	**-0.125**	**-0.093**	**-0.114**	**0.043**
	A	2	2	3	2	3	4	
MHco3.N2.F_2	N	17	*	28	28	16	14	
	H_e	0.392	*	0.636	0.499	0.653	0.754	
	H_o	0.118	*	0.464	0.286	0.438	0.786	
	P-value	0.002	*	0.006	0.047	0.224	0.515	
	F_{is}	**0.706**	*****	**0.274**	**0.432**	**0.338**	**-0.044**	**0.34**
	A	3	*	3	2	3	4	
MHco3	N	54	54	46	56	56	54	
	H_e	0.736	0.475	0.551	0.467	0.523	0.681	
	H_o	0.667	0.593	0.607	0.5	0.5	0.407	
	P-value	0.008	0.236	0.874	1	0.657	0.013	
	F_{is}	**0.096**	**-0.243**	**-0.104**	**-0.071**	**0.044**	**0.406**	**-0.088**
	A	4	2	3	2	3	5	

N: number of individuals genotyped. H_e: expected heterozygosity. H_o: observed heterozygosity.
F_{is}: inbreeding coefficient. A: number of alleles. * monomorphic locus: not tested.

Table 4.7: Population genetic data for six microsatellite loci (8a20, Hcms36, 3561, X182, X256 and Hcms25) on the F_1 and F_2 populations of the N1 and N2 inbred lines and the parent MHco3 population.

4.3.2.3.5 Pairwise Fst analyses

The population pairwise Fst values comparing the F_1 and F_2 populations of the MHco3.N1 and MHco3.N2 inbred lines and of the parent MHco3 population are shown in Table 4.8. The F_1 and F_2 populations of the same inbred lines (F_1 vesus F_2 for MHco3.N1 and F_1 versus F_2 for MHco3.N2) are very genetically similar (Fst values <0.05) as should be the case. In contrast the F_1 populations of the MHco3.N1 and MHco3.N2 lines are extremely genetically divergent (Fst value 0.41). The F_1 population of the MHco3.N1 line is much more similar to the parental MHco3 (ISE) population (Fst value <0.1) than is the F_1 population of MHco3.N2 line (Fst value 0.22). This suggests that the founding parent(s) of the MHco3.N2 line had a more divergent genotype with respect to the general MHco3 (ISE) population than the MHco3.N1 line. In other words, the N2 female (or male mate) had a number of alleles present at the loci examined that were rare in the general MHco3 (ISE) population.

	MHco3.N1.F_1	MHco3.N1.F_2	MHco3.N2.F_1	MHco3.N2.F_2	MHco3
MHco3.N1.F_1	0				
MHco3.N1.F_2 (P-value)	0.04537 (0.027)	0			
MHco3.N2.F_1 (P-value)	0.41306 (<0.001)	0.25650 (<0.001)	0		
MHco3.N2.F_2 (P-value)	0.33334 (<0.001)	0.17358 (<0.001)	0.04749 (0.054)	0	
MHco3 (P-value)	0.07933 (<0.001)	-0.00597 (0.55)	0.20777 (<0.001)	0.11357 (<0.001)	0

Table 4.8: Population pairwise FSTs for six microsatellite loci (8a20, Hcms36, 3561, X182, X256 and Hcms25) comparing the F_1 and F_2 populations of the MHco3.N1 and MHco3.N2 inbred lines and the parent MHco3 population.

4.4 Discussion

4.4.1 Successful development of a method for single *H. contortus* pair mating

The results in the previous chapter demonstrated that attempts to recover the progeny of a mating between a single male and a single female adult *H. contortus* following their direct surgical transfer of into the abomasum of a recipient sheep were unsuccessful. The preliminary studies identified problems such as: the improbability of single nematode parents both surviving, finding each other and mating within the abomasum of a sheep; concerns about parasitic nematode contamination of recipient sheep; and issues caused by fly-borne parasitic nematode contamination of coprocultures. However, these preliminary studies provided valuable information to allow an alternative approach to be developed. They identified the importance of using day 14, sexually immature *H. contortus* for surgical transfer into recipient sheep and the advantages of using young donor and recipient lambs during the spring and summer months. They also showed that simple parasitological data in isolation can be misleading and that genetic validation is required to be certain that a single pair mating or genetic cross has been successful.

This chapter has presented the development of an alternative method to achieve single pair mating involving surgical transfer of 20 female and one male day 14 *H. contortus* to increase the probability of the male finding a female. The modified experimental protocol took into account the polyandrous mating behaviour of *H. contortus* by using a single male. It also addressed concerns about parasitic nematode contamination of recipient sheep by euthanasing them and recovering egg laying female *H. contortus* within the minimum prepatent period of contaminant parasitic nematodes. Issues caused by fly-borne parasitic nematode contamination of coprocultures were addressed by their incubation in an isolated closed environment. The genetic analysis presented in this chapter has demonstrated that this modified approach to single pair mating was successful.

Failure to recover the male parent *H. contortus* from any of the three recipient lambs, while between 30% and 60% of the females were recovered was disappointing as the determination of both parental genotypes founding the inbred lines would have aided genetic validation. This might suggest that the behaviour of male parasitic nematodes in seeking out females predisposes to their loss from the abomasum. The results of the previous chapter suggest that female *H. contortus* can store spermatozoa and produce viable progeny for a significant period of time after mating. Therefore, since none of the adult female nematodes recovered from the abomasum of recipient lamb C produced viable progeny *in vitro*, it seems likely that none had mated successfully, perhaps due to loss of the male *H. contortus* soon after surgical transfer. By the same logic, the male *H. contortus* that was transferred into recipient lamb A may only have mated with one surviving female before being lost, while the male that was transferred into recipient lamb B would have mated with at least 3 females.

The importance of using ITS2 and NTS molecular markers to confirm the absence of non *H. contortus* contamination was demonstrated during the preliminary studies (Chapter 3). The methods used to prevent parasitic nematode contamination of the recipient lambs and F_1 coprocultures were apparently effective, involving early euthanasia of the recipient lambs, and performance of coprocultures in a closed environment, primarily on filter paper in a live *E. coli*/LB broth. However, it is possible that the low level of genetic drift that was observed in the inbred F_2 and F_3 populations could have arisen due to *H. contortus* contamination of the donor sheep or coprocultures, although this cannot be proven.

The recovery of 7 adult female and 3 adult male *H. contortus* (F_1 progeny of the single parent mating) from the abomasum of the MHco3.N2 F_2 donor lamb, 148 days after infection with an estimated 10 female and 10 male L_3 is noteworthy. Each of the female *H. contortus* would have shed between 3,000 and 4,000 eggs per day between about days 18 and 64 after L_3 infection. These figures are higher than those deduced from

other infections involving larger numbers of *H. contortus* (Chapters 3 and 5), implying an adaptive strategy to ensure the survival of very low parasite numbers (or that a loss of fecundity occurs associated with large parasite numbers) (references could not be found in support of this discussion point, re-enforcing the fact that current understanding of the biology of sheep parasitic nematodes is rudimentary. Similar findings have previously been observed (Frank Jackson, *personal communication*). Differences in the FWECs of donor lambs infected with the same numbers of F_2 and F_3 *H. contortus* could have arisen due to differences in the ages of the donor lambs used and of the times of year when the donor lambs were infected (Chapter 3). The large difference in FWECs between the two donor lambs infected with similar numbers of MHco3.N1 F_3 *H. contortus* L_3 could have been associated with similar nematode density dependent effects. Unfortunately, these confounding factors prevented the direct comparison of nematode survival and egg production between the filial generations of each inbred *H. contortus* line.

4.4.2 Egg development and hatching

Identification of a low rate of egg hatching and development in the F_2, F_3 and F_4 generations of one inbred line, but not in the other might indicate the inadvertent selection for a small number of lethal genetic mutations in the affected MHco3.N1 line. (If mutations at more than a few loci were involved, they might have been expected to have been present in both inbred lines.) However, if the low rate of egg hatching was determined by a single, recessive allele, then the frequency of that allele in the inbred filial generations would have been 25% or 50% depending on whether it was present in one or both parents, and the frequency of homozygous lethal genotypes in the F_2 generation would have been much lower than the observed lethal phenotypic frequency of about 70%. Furthermore, if failure of egg hatching was determined by a single recessive allele, the phenotypic frequency would be reduced in the F_3 and F_4 generations, because homozygotes would not survive in the populations. Such a reduction in frequency was observed in the MHco3.N_1 F_3 and MHco3.N1 F_4

generations, but these results could have been confounded by effects of lamb donor age and the times of year of donor lamb infections on *H. contortus* survival and egg production, for example if the lethal mutation was linked to genes conferring general fitness in these circumstances. Furthermore, the frequency of the failure of egg hatching phenotype was unchanged between the MHco3.N1 F_2 and MHco4.N1 $F_4(2)$ *H. contortus* populations.

Observations throughout the course of this study show that the proportion of MHco3 (ISE) *H. contortus* eggs that normally do not hatch is about 3%, which is higher than the proportion of eggs that do not hatch in the free-living model nematode *C. elegans* (John Gilleard, *personal communication*). This difference is possibly a consequence of an extremely large level of genetic variation in *H. contortus* which might lead to an increase in the frequency of deleterious mutations compared with *C. elegans*. Only 6.5% of the MHco3 (ISE) *H. contortus* eggs that did not hatch had arrested development as single cell oocytes, implying that fertilisation is highly efficient, while 76.6% of unhatched eggs had arrested development as pre-hatch larvae, presumably due to abnormal karyotypes. By contrast, 89.5% of the inbred MHco3.N1 $F_4(2)$ eggs arrested development pre-morphogenesis. The heterozygote deficiency that was observed in these eggs might indicate that some had not been fertilised, although this situation is unlikely, because almost all of the adult MHco3.N1 F_3 *H. contortus* female parents were shown to have mated by their shedding of some fertilised eggs *in vitro*. The difference between the MHco3 (ISE) and MHco3.N1 $F_4(2)$ *H. contortus* in the stage at which most egg development was arrested therefore implies the presence of a higher frequency of specific lethal mutations in the inbred line.

The presence of females with no eggs and depression of L_3 yields of coprocultures was previously reported during the production of the Hco3 (ISE) strain of *H. contortus* after a few generations of half-sibling inbreeding (Roos and others, 2004). This problem was overcome by infecting a donor with a mixture of 1000 – 5000 L_3 from a previous generation (Roos and others, 2004). The mechanisms of reduced egg developmental

success caused by inbreeding might involve multiple forms of variations in chromosome structure or aneuploidy, for example associated with non disjunction and pairing between non homologous chromosomes (Le Jambre and Royal, 1980), or an increased frequency of deleterious mutations. Further study of egg development and hatching in inbred parasitic nematodes would be of interest, because if it is determined by a small number of major lethal genetic mutations, then the clarification of its molecular basis could lead to the development of a potential target mechanism for nematode control. In the first instance, cytological studies of testes and ova from the inbred adult *H. contortus*, to identify karyotype abnormalities in the gametocytes could help to clarify the mechanisms of reduced developmental success.

4.4.3 *In vitro* bioassays to characterise the inbred *H. contortus* lines

The EHA and LFIA dose response curves for the inbred lines were indistinguishable from those for MHco3 (ISE) *H. contortus*, apart from the influences of poor MHco3.N1 egg hatching in the drug free controls for the EHA and the lower percentages of inbred F_3 and F_4 L_1 feeding in drug free control replicates, which was probably associated with the time of year (December and January) when the assays were performed. These findings demonstrate that the inbreeding through single parent genetic crosses did not inadvertently select for benzimidazole or macrocyclic lactone anthelmintic resistance.

4.4.4 Molecular genetic evidence for successful inbreeding

This study has shown that the isotype 1 β-tubulin F200Y (*T/A*) SNP (Kwa and others, 1994) is present in about 5% of MHco3 (ISE) *H. contortus*, despite the strain having been inbred from homozygous (*TT*) female *H. contortus* (Roos and others, 2004). This situation could have arisen through the use of a heterozygous (*AT*) male or by the subsequent contamination of donor lambs or coprocultures. The absence of the F200Y (*T/A*) SNP in both inbred lines supports the premise that they are derived from a single

pair mating and also provides a potentially useful resource for comparisons between benzimidazole susceptible and resistant *H. contortus* and detailed phenotypic studies of benzimidazole resistance.

A high level of polymorphism was shown to be present in the GABA Cl subunit HG1 (Blackhall and others, 2003) of the MHco3 (ISE) *H. contortus*, caused at least in part by the presence of SNPs within the gene. Demonstration of at least 5 different haplotypes in the progeny of a single female *H. contortus*, was consistent with the existing evidence for polyandry based on X chromosome and autosomal microsatellite genotypes (Redman and others, *In Press*), and reaffirmed the high level of polymorphisms within the males. The GABA Cl SSCP showed a clear loss of polymorphism in the F_1 and F_2 progeny of the MHco3.N1 female *H. contortus*, with only 3 different genotypes judged to be present, providing strong support for successful inbreeding.

Estimates of the microsatellite alleles that were present in Genescan traces produced from bulk DNA lysates of about 500 nematodes were in broad agreement with the results obtained from individual nematodes, albeit that the bulk Genescan traces did not identify alleles that were present at a low frequency. The bulk Genescan traces and individual genotyping showed a loss of alleles at various microsattelite loci in the two inbred lines, demonstrating the success of inbreeding. As would be predicted if the two inbred lines were founded by single pair matings, both the bulk Genescan traces and the individual genotyping showed selection for different alleles in the two inbred lines.

The individual microsatellite genotypes of the adult female parent and inbred filial generation nematodes enabled the application of Mendelian principles in support of a successful single parent genetic cross. For example, if the N1 and N2 parental female genotypes at an autosomal chromosome microsatellite locus were *a.a* and *a.b*, then the genotypes of the male parents could be predicted. If both male parents had been *a.a*, then the a:b allele frequencies in the N1 and N2 F_1 progeny would have been 1:0 and 3:1 respectively, while if they had both been *a.b*, then the a:b frequencies in the N1 and N2

F_1 progeny would have been 3:1 and 1:1 respectively. Assuming Hardy Weinberg equilibrium, then the same allele frequencies would be present in the F_2 generation. Hence if single pair mating has been successful, one would expect not just a loss of polymorphism, but also the allele frequencies should approximate to these simple Mendelian predictions in the resulting lines (as opposed to a variety of high and low frequencies). Overall the results do indeed fit these Mendelian predictions well and are consequently entirely consistent with a single pair mating. This is in contrast to the results in the previous chapter. In reality, while these principles enabled the genotypes of the males to be predicted at most loci, the allele frequencies in the inbred filial generations only approximated the predicted values. This inaccuracy was probably caused by allelic dropout in certain PCRs of DNA lysates prepared from L_3, rather than failure of successful inbreeding.

The individual nematode microsatellite data enabled the calculation of inbreeding coefficient (F_{is}) values which showed the inbred F_2 populations to be more inbred than the reference MHco3 population, from which they were derived. However the results for the F_1 populations were less clear. Errors in the calculations of F_{is} indices for the F_1 populations may have arisen for several reasons, including: possible confounding effects of allelic dropout; differences in the inherent polymorphism, reliability and weakness of individual markers; the contribution of a monomorphic marker; or differences in the efficiency of the microsatellite PCR for heterozygous loci between L_1 (which comprised most of the F_1 genotypes) and adults or L_3 (which comprised most of the F_2 genotypes). Furthermore, the H_e and H_o values for the two X chromosome loci will be inaccurate, because the calculations fail to take into account the fact that male nematodes only have one allele at these loci. However, if an assumption is made that these errors will be consistent between each population, then comparison of F_{is} values for MHco3.N1 and MHco3.N2 with MHco3 should remain valid and supports the premise for successful inbreeding of the MHco3 (ISE) strain of *H. contortus*.

The microsatellite data show selection for different alleles in the two inbred lines, as demonstrated in the multilocus genotyping PCA plots and pairwise Fst analyses. The production of genetically divergent inbred *H. contortus* lines in this way could be exploited for the production of a genetic map, to identify the position of genes and neutral molecular markers relative to each other (Le Jambre and others, 1999b). The method of producing genetically divergent inbred lines could also have applications to segregate different aspects of anthelmintic resistance, to enable comparative studies of candidate gene SNPs, and to test genetic markers for anthelmintic resistance.

Chapter 5: Development of a method to backcross ivermectin resistance-conferring genes into a susceptible *H. contortus* genetic background

5.1 Introduction

Most research aimed at identifying molecular markers for anthelmintic resistance has focussed on the investigation of possible associations between candidate genes, selected on the basis of knowledge of the anthelmintic drug's mode of action, and the resistance phenotype. Although this approach has been successful for benzimidazole resistance, to-date it has not identified a major single molecular marker for macrocyclic lactone resistance. This might indicate the involvement of genes that have hitherto not been found or recognised as being associated with drug action, or that the mechanism of resistance does not involve a single major determinant. Even if mutations are identified in candidate genes, provision of conclusive proof of a causal relationship with the resistance phenotype is complex. For example, comparison of the frequency of the candidate polymorphism between populations of differing drug susceptibility may not be conclusive due to the extensive genome-wide genetic variation that exists between isolates. One standard approach to overcome this problem is to experimentally select for anthelmintic resistance from a susceptible isolate, and then to compare the anthelmintic susceptible and resistant lines derived from the single isolate. However this approach has limitations because loci in addition to those responsible for resistance may be selected, for example those conferring general fitness traits or enhanced fecundity. Furthermore, mechanisms identified as a result of artificial selection for resistance might not reflect those that are important in field populations. Finally, when a resistance-conferring mutation is selected by anthelmintic treatement, selection also acts on

genetically linked regions of the genome, with these linked associations being broken down over time by recombination. Therefore, an apparent association between a genetic polymorphism and the resistance phenotype does not in itself prove causality. Current understanding of recombination rates and the extent of linkage disequilibrium in different regions of the *H. contortus* genome in different situations is insufficient for simple association studies of candidate gene polymorphisms and anthelmintic resistance phenotype to be interpreted with confidence (Gilleard and Beech 2007). Consequently, there is a need to develop different experimental approaches to identify resistance genes, for example genetic approaches that can overcome some of the limitations outlined above.

The classical genetic approach outlined in chapter 3, is to undertake a genetic cross between a single nematode from a field selected resistant isolate and a single nematode from a characterised susceptible isolate. If the F_2 progeny of such a cross can then be categorised as anthelmintic resistant and susceptible, then genome-wide approaches can be used to map loci linked to the resistance-conferring polymorphisms. Unlike conventional candidate gene studies that are usually based on knowledge of specific mechanisms of drug action, this approach does not rely on pre-conceived ideas about putative resistance genes of interest and therefore, also potentially identifies genes conferring anthelmintic resistance through novel gene regulatory and xenobiotic defence mechanisms. The approach ultimately needs a fully sequenced and annotated *H. contortus* genome and the ability to genotype markers across the genome quickly and efficiently. The original aim of this study was to perform such a genetic cross, with the view that although the genome resources were still incomplete, material could be archived and analysed in an iterative fashion as resources and technologies improve. However, a number of technical difficulties with this approach were identified. Firstly, the single pair matings described in chapter 3 were not successful, although this problem could be solved by using the modified approach described in chapter 4. Secondly, accurate determination of the anthelmintic resistance phenotype of individual worms was problematic. This is a critical requirement of the approach that remains a constraint

to the single worm genetic cross and F_2 progeny analysis approach. Hence a different genetic approach, involving mass matings was devised and is the subject of this chapter. This approach involves matings between populations of male worms of one strain with females of the other and determination of the resistance phenotype on a population basis.

The concept of the mass mating genetic approach is to backcross regions of the genome containing anthelmintic resistance-conferring polymorphisms from a resistant strain (derived from a natural field isolate) into the genetic background of a well characterised susceptible isolate. This can be achieved by first performing a genetic cross between populations of the susceptible and resistant strains and then selecting for phenotypically resistant nematodes in the F_1 progeny population by treating *in vivo* with a dose of the anthelmintic that will kill susceptible worms. Resistant F_1 progeny can then be backcrossed with the parental susceptible population and the process repeated a number of times with anthelmintic selection applied at each generation. The genetic backround of the resistant parental isolate should be reduced by 50% at each backcross such that F_1, first, second, third and fourth generations should have 50%, 75%, 87.5%, 93.7% and 96.9% of their genetic background derived from the parental susceptible isolate. However, resistance genes (and linked regions of the genome) should be retained in these backcross progeny. Hence after five generations of genetic crossing and backcrossing, the resulting lines should effectively consist of the parental susceptible genetic background apart from regions of the resistant parental isolate surrounding the resistance gene or genes (Fig 5.1).

(Note: references to backcrossing experiments in parasitic nematodes are provided in the discussion of this chapter, while references to genetic crossing experiments in protozoal parasites are provided in Chapter 6.)

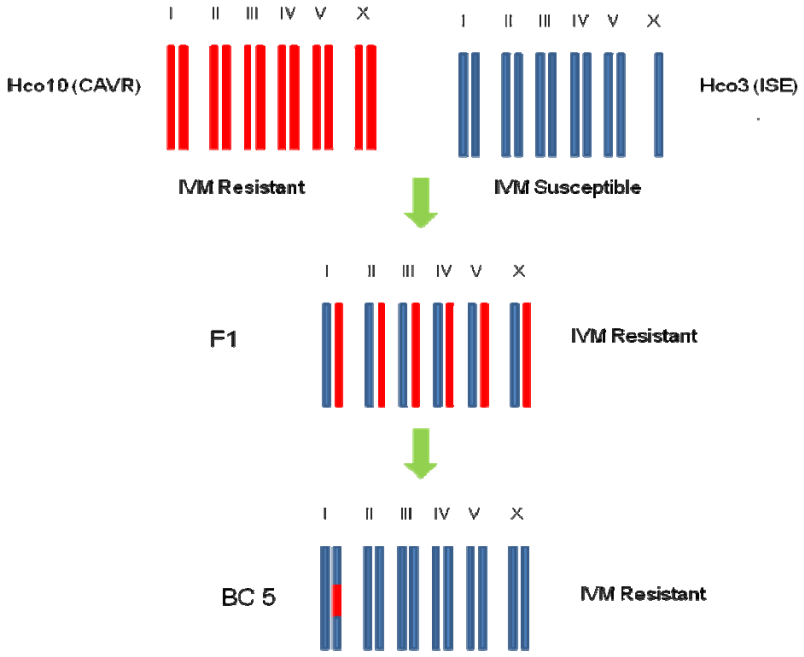


Fig 5.1: The premise of the genetic backcrossing approach to identify genes conferring ivermectin resistance and surrounding regions of the genome (diagram courtesy of John Gilleard).

If a population of worms from such a backrossed line are genotyped with genome-wide markers, then the alleles present (and their frequencies) should be characteristic of the susceptible parental strain at the majority of loci. However, for those regions of the genome linked to the resistance-conferring loci the alleles present (and their frequencies) should be characteristic of the resistant parental strain. Hence, regions of the genome linked to the resistance-conferring polymorphisms can be identified. In principle, the greater the number of backcross generations, the more tightly the locus mapped by this approach will become, although the available genomic resources and understanding of recombination rates in different parts of the *H. contortus* genome are currently insufficient to accurately locate the locus. However, analysis of even a few hundred appropriate markers could identify the region(s) of the genome in which the resistance-conferring polymorphism(s) reside. As genomic resources, markers, genotyping technologies and understanding of the genetics of the organism improve, then more detailed and accurate analysis can be applied. The work outlined in this chapter

represents a proof of concept study with the aim of producing backcrossed lines, from which material can be archived for future iterative analysis.

5.2 Materials and methods

5.2.1 Strains of *H. contortus*

The strains of *H. contortus* used in this study are all maintained at the Moredun Research Institute by passage through donor sheep. The MHco3 (ISE) strain was used as the parent ivermectin susceptible population (this strain has also been characterised as susceptible to imidazothiazole and benzimidazole anthelmintics). This strain is being used as template for the *H. contortus* genome project. The MHco4 (White River/WRS) strain was used as a field selected ivermectin resistant strain (this strain has also been characterised as susceptible to imidazothiazole and resistant to benzimidazole anthelmintics (Roos and others, 2004; Dave Bartley, *personal communication*). The MHco10 (Chiswick avermectin resistant/CAVR) strain was used as a genetically divergent ivermectin resistant strain (this strain has also been characterised as susceptible to imidazothiazole and benzimidazole anthelmintics (Le Jambre, 1993; Dave Bartley, *personal communication*).

The F_1 progeny of the first genetic crosses were designated as MHco3/4 and MHco3/10 and the subsequent backcrosses were designated as MHco3/4.BC_n and MHco3/10.BC_n, denoting the Moredun Research Institute (M), *H. contortus* (Hco), the unique numbers allocated to both parental isolates (3/4 or 3/10), and the backcross generation ($BC_{2, 3 \text{ and } 4}$).

5.2.2 Surgical transfers

5.2.2.1 Preparation of worm free donors and recipients

Male worm free donor and recipient lambs were treated sequentially with 5 mg/kg of fenbendazole (Panacur 2.5%; Intervet) and 7.5 mg/kg of levamisole (Levacide 3%; Norbrook) 7 to 14 days before oral infection with L_3 or surgery.

5.2.2.2 Genetic crosses between parental strains

Three male worm free donor lambs were orally dosed on day 0 with about 10,000 parent MHco3 (ISE), MHco4 (WRS), or MHco10 (CAVR) *H. contortus* L_3. The donor sheep were euthanased on day 14 post infection. Fifty male late L_4/immature adult MHco3 (ISE) *H. contortus* and 50 female late L_4/immature adult MHco4 (WRS) *H. contortus* were then surgically transferred into the abomasum of a male worm-free recipient lamb, within about 1 hour of recovery from the donor sheep. Fifty male late L_4/immature adult MHco3 (ISE) *H. contortus* and 50 female late L_4/immature adult MHco10 (CAVR) *H. contortus* were surgically transferred into the abomasum of a male worm-free recipient lamb, within about 1.5 hours of recovery from the donor sheep. Harnesses and bags were fitted to the recipient lambs from 3 days after surgery to enable faecal collection and coproculture of the MHco3/4 and MHco3/10 progeny of the genetic crosses to L_3.

FWECs were performed 3 or 4 times weekly using a modification of the cuvette method described by Christie and Jackson (1982). Supportive evidence of the identity of eggs as those of *H. contortus* was periodically obtained by fluorescent agglutinin staining. Confirmation of the identity of L_3 recovered from coprocultures as *H. contortus* was periodically obtained by examination of larval morphology. Bulk and individual nematode DNA lysates were made and PCRs performed using primers flanking the ITS2 and NTS loci. The recipient sheep were maintained until their FWECs fell to a consistently low level, when they were euthanased, and any surviving nematodes were recovered from their abomasa.

5.2.2.3 Backcrosses

Male worm free donor lambs were orally dosed on day 0 with between 5,000 and 10,000 MHco3 (ISE), MHco3/4.BC_n, or MHco3/10.BC_n *H. contortus* L_3. The MHco3/4.BC_n and MHco3/10.BC_n *H. contortus* donors were treated with a half therapeutic dose of 100 μg/kg of ivermectin (Oramec drench for sheep; Merial) on day 10 or 11 post infection. The donor sheep were euthanased on day 14 post infection to select for ivermectin resistant progeny and 45 to 100 male late L_4/immature adult MHco3 (ISE) *H. contortus* and 50 to 100 female late L_4/immature adult MHco3/4.BC_n, or MHco3/10.BC_n *H. contortus* were then surgically transferred into the abomasa of male worm free recipient lambs, within 2 hours of recovery from the donor sheep. Harnesses and faecal collection bags were fitted to the recipient lambs from 3 days after surgery to enable faecal collection and coproculture of the progeny of the backcrosses to L_3. The protocol is summarised in Fig 5.2.

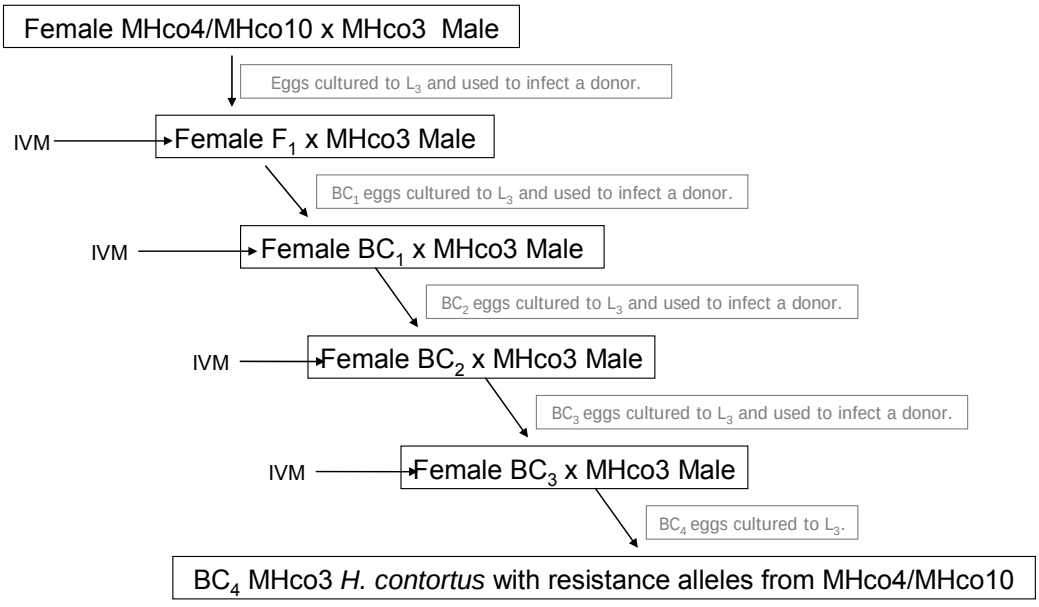


Fig 5.2: Summary of the backcross protocol and terminology used in this chapter. (IVM: treatment with ivermectin.)

FWECs were performed routinely and supportive evidence of the identity of eggs as those of *H. contortus* was periodically obtained by fluorescent agglutinin staining, as for the first genetic cross. Confirmation of the identity of L_3 recovered from coprocultures as *H. contortus* was periodically obtained by examination of larval morphology and using ITS2 and NTS molecular markers. The recipient sheep were maintained until their FWECs fell to a consistently low level, or until they were no longer required, when they were euthanased, and nematodes were recovered from their abomasa.

5.2.2.3.1 Confirmation of efficacy of a half therapeutic dose of ivermectin

A donor sheep was infected with 10,000 MHco3 (ISE) *H. contortus* and treated with a half oral dose of 100 μg/kg ivermectin (Oramec drench for sheep; Merial) on day 11 post infection. FWECs were performed from day 18 post infection and the sheep was killed and a post-mortem abomasal nematode count was performed on day 22 post infection. A second donor sheep was infected with 5,000 MHco3 (ISE) *H. contortus* and treated with a half oral dose of 100 μg/kg ivermectin (Oramec drench for sheep; Merial) on day 21 post infection. Pre and post treatment FWECs were monitored and the sheep was killed and a post-mortem abomasal nematode count was performed on day 32 post infection.

Evidence of lack of total efficacy of a half oral dose of 100 μg/kg ivermectin against the genetic cross (F_1) and backcross progeny was afforded by their survival in the donor lambs used in the backcross experiment. Furthermore, a faecal egg count reduction test was performed using a single donor sheep that had been infected with 8000 L_3 cultured from the MHco3/10.BC2 recipient. The sheep was treated with 100 μg/kg ivermectin (Oramec drench for sheep; Merial) on day 30 post infection. Pre and post treatment FWECs were monitored and the sheep was killed and a post-mortem abomasal nematode count was performed on day 32 post infection.

5.2.3 Characterisation of the backcross progeny

5.2.3.1 In vitro *bioassays*

Egg hatch assays and larval feeding inhibition assays were performed on the parent MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus* and on the progeny of the first genetic cross and subsequent backcrosses. Egg hatching and larval feeding was assayed in concentrations of 0 to 0.5 µg/ml thiabendazole and 0 to 0.33 µg/ml of ivermectin respectively. ED_{50} (egg death), LFI_{90} and LFI_{99} (larval feeding inhibition) values were calculated by Probit analysis, using Minitab ver.13 software.

5.2.3.2 Use of microsatellite markers to demonstrate the success of the backcrosses

A panel of microsatellite markers was used to validate and monitor the backcrosses. The markers were amplified from bulk DNA lysates of the parental, genetic cross (F_1) and backcross populations. Determination of the alleles that are present in DNA lysates made from mass populations of worms and estimation of their respective frequencies were made by the *area under the curve* method that has been previously validated (Redman and others, 2008). Lysates were made of pools of about 200 day 14 late L_4/immature adult MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) *H. contortus* and bulk preparations of about 500 L_3 recovered from coprocultures of the progeny of the first genetic crosses and subsequent backcrosses. Two independent lysate preparations were made from each nematode generation. Primer pairs flanking a panel of 19 polymorphic and diverse microsatellite loci were used to PCR amplify the selected loci. The markers used were: 8a20; 22co3; 3561; 18210; 26981; 40506; 60435; 181881; Hcms25; Hcms27; Hcms33; Hcms36; Hcms40; HcmsX142; HcmsX146; HcmsX151; HcmsX182; HcmsX256; and HcmsX337. PCRs and capillary sequencing in conjunction with Genescan software were then used to generate chromatograms for each bulk lysate. The chromatograms were analysed using GeneMapper software. The frequencies of the alleles that were present were estimated from the relevant peak heights on the Genescan trace, to enable comparison of the parent and progeny population genetic fingerprints.

5.2.3.3 Efficacy of a full dose of ivermectin

A donor lamb was infected with 5,000 MHco3/4.BC_4 and another with 5,000 MHco3/10.BC_4 L_3. These sheep were treated with a full therapeutic oral dose of 200 μg/ml ivermectin (Oramec drench for sheep; Merial) on day 29 post infection. Pre and post treatment FWECs were monitored and postmortem total abomasal nematode counts were performed.

5.2.4 Archiving of material

Genomic DNA from parent and progeny nematodes was stored at -80^{o}C as bulk lysates. Surplus late L_4/immature adult *H. contortus* were recovered from each of the donor animals on the day of surgical transfer were stored in 100% ethanol. Aliquots of 50,000 or 100,000 L_3 progeny of the first genetic crosses and each of the backcrosses recovered from coprocultures were cryopreserved in liquid nitrogen. Any adult *H. contortus* recovered from donor or recipient sheep were stored in 100% ethanol

5.3 Results

5.3.1 Backcrosses

Genetic crosses and backcrosses were performed for each isolate to produce fourth backcross populations of MHco3/4.BC_4 and MHco3/10.BC_4 strains of *H. contortus*. An indeterminable proportion of the progeny of the first genetic crosses and of each subsequent first, second and third backcross survived treatment with a half therapeutic dose of 100 μg/kg of ivermectin. Worms surviving treatment should have genes derived from the parent MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus* that confer phenotypic resistance to a half dose of 100 μg/kg ivermectin.

The success of establishment of infections in donor sheep was variable and influenced the number of late L_4/early adult nematodes that could be picked and surgically transferred within 2 hours of killing the donor sheep. The yields of the donors used for the parent and second backcross generations were lowest, these animals being about 11 months-old and 10 months-old, respectively. The highest yields were obtained from the youngest donors, which were about 3 months-old. Some rounds of donor infection yielded insufficient late L_4/immature adult *H. contortus* for surgical transfer. These unsuccessful infections involved at different stages the production of MHco3 (ISE) and MHco3/10.BC_2 populations, and were putatively related to undetermined effects of the time of year, age of the donors and the immune status of the donors. Table 5.1 shows the dates of the successful completion of different procedures involved with the backcrossing experiments, numbers of L_3 used to infect donor sheep and numbers of late L_4/immature adults that were surgically transferred to recipients. The FWECs of the recipients and numbers of adult nematodes recovered from their abomasa after they were killed varied throughout the experiment, influenced partly by the number of male and female *H. contortus* that were transferred, and also with the age of the donors and time of year. FWEC data and numbers of adult male and female *H. contortus* that were recovered from the recipient sheep after they were killed are presented in Fig 5.3. Female *H. contortus* generally survived for longer than male *H. contortus*.

Date	Infection of donors	100 µg/kg IVM treatment	Surgical transfer		Progeny
			♂	♀	
6-3-07	10,000 MHco3 10,000 MHco4 10,000 MHco10				
20-3-07			50 MHco3 50 MHco3	50 MHco4 50 MHco10	MHco3/4 MHco3/10
23-7-07	5,000 MHco3 5,000 MHco3/4 5,000 MHco3/10				
2-8-07		MHco3/4 MHco3/10			
6-8-07			70 MHco3 50 MHco3	90 MHco3/4 65 MHco3/10	MHco3/4.BC_1 MHco3/10.BC_1
17-9-07	8,000 MHco3 8,000 MHco3/4.BC_1 8,000 MHco3/10.BC_1				
27-9-07		MHco3/4.BC_1 MHco3/10.BC_1			
1-10-07			45 MHco3 45 MHco3	55 MHco3/4.BC_1 55 MHco3/10.BC_1	MHco3/4.BC_2 MHco3/10.BC_2
30-11-07	10,000 MHco3 10,000 MHco3/4.BC_2				
11-12-07		MHco3/4.BC_2			
14-12-07			100 MHco3	75 MHco3/4.BC_2	MHco3/4.BC_3
7-4-08	10,000 MHco3 10,000 MHco3/4.BC_3 10,000 MHco3/10.BC_2				
18-4-08		MHco3/4.BC_3 MHco3/10.BC_2			
21-4-08			100 MHco3 100 MHco3	100 MHco3/4.BC_3 100 MHco3/10.BC_2	**MHco3/4.BC_4** MHco3/10.BC_3
4-6-08	10,000 MHco3 10,000 MHco3/10.BC_3				
15-6-08		MHco3/10.BC_3			
18-6-08			85 MHco3	90 MHco3/10.BC_3	**MHco3/10.BC_4**

Table 5.1: Dates of procedures in the backcross experiments and numbers of L_3 (infection of donors) and late L_4/immature adults (surgical transfer) dosed or transferred.

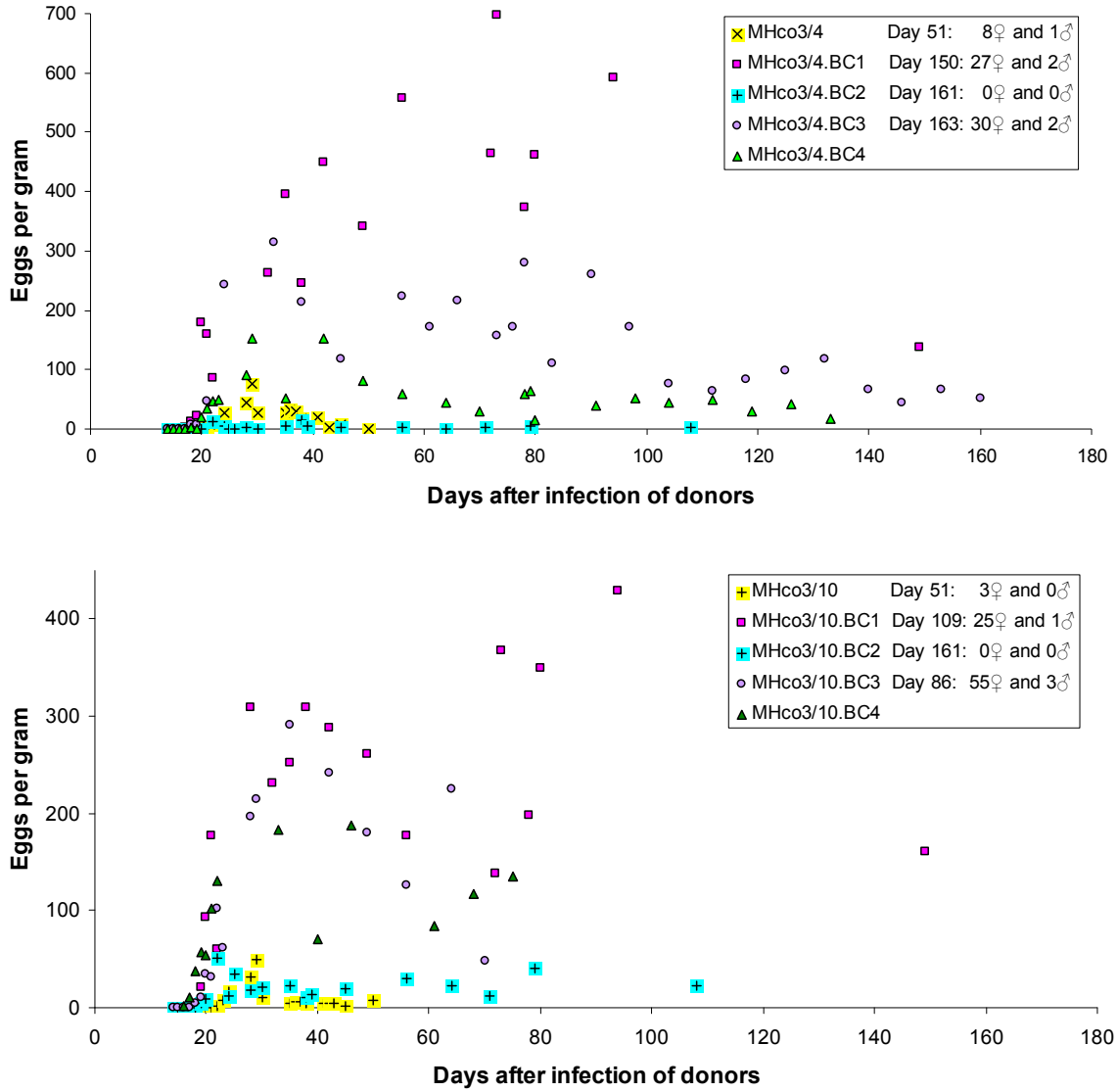


Fig 5.3: FWECs of the recipient sheep and numbers of female and male *H. contortus* recovered postmortem from their abomasa.

Support of the identity of the progeny of the genetic crosses and backcrosses as being *H. contortus* was provided by the morphological appearance of sheathed and exsheathed L_3 and positive fluorescence of the shells of eggs stained with a fluorescent peanut agglutinin. The identity of the progeny of the genetic crosses and backcrosses as *H. contortus* was supported by identification of 325 bp and about 1.5 kb products of genus and species specific ITS2 and NTS PCRs.

5.3.2 In vitro bioassays

5.3.2.1 Egg hatch assays

Egg hatch assays were performed on two or three separate occasions on the progeny of each genetic cross and backcross (with the exception of MHco3/4.BC_2 and MHco3/10.BC_3) and on eggs recovered from contemporary MHco3 (ISE) donor sheep. Assays could not be performed on MHco3/4.BC_2 eggs, because the FWEC of the recipient sheep was too low to yield sufficient eggs and results that are shown for MHco3/10.BC_3 are unreliable, because they are based on eggs that had to be extracted form faeces taken form an overnight collection, rather than directly from the rectum.

The dose response curves produced for the MHco3/10 genetic cross (F_1 generation) and for each backcross were indistinguishable from those produced for the benzimidazole susceptible MHco3 strain of *H. contortus*. However, the dose response curves produced for the MHco3/4 genetic cross (F_1 generation) differed, demonstrating phenotypic benzimidazole resistance. Dose response curves, based on the mean percentage of hatched eggs in each assay (± standard deviations) are shown in Fig 5.4.

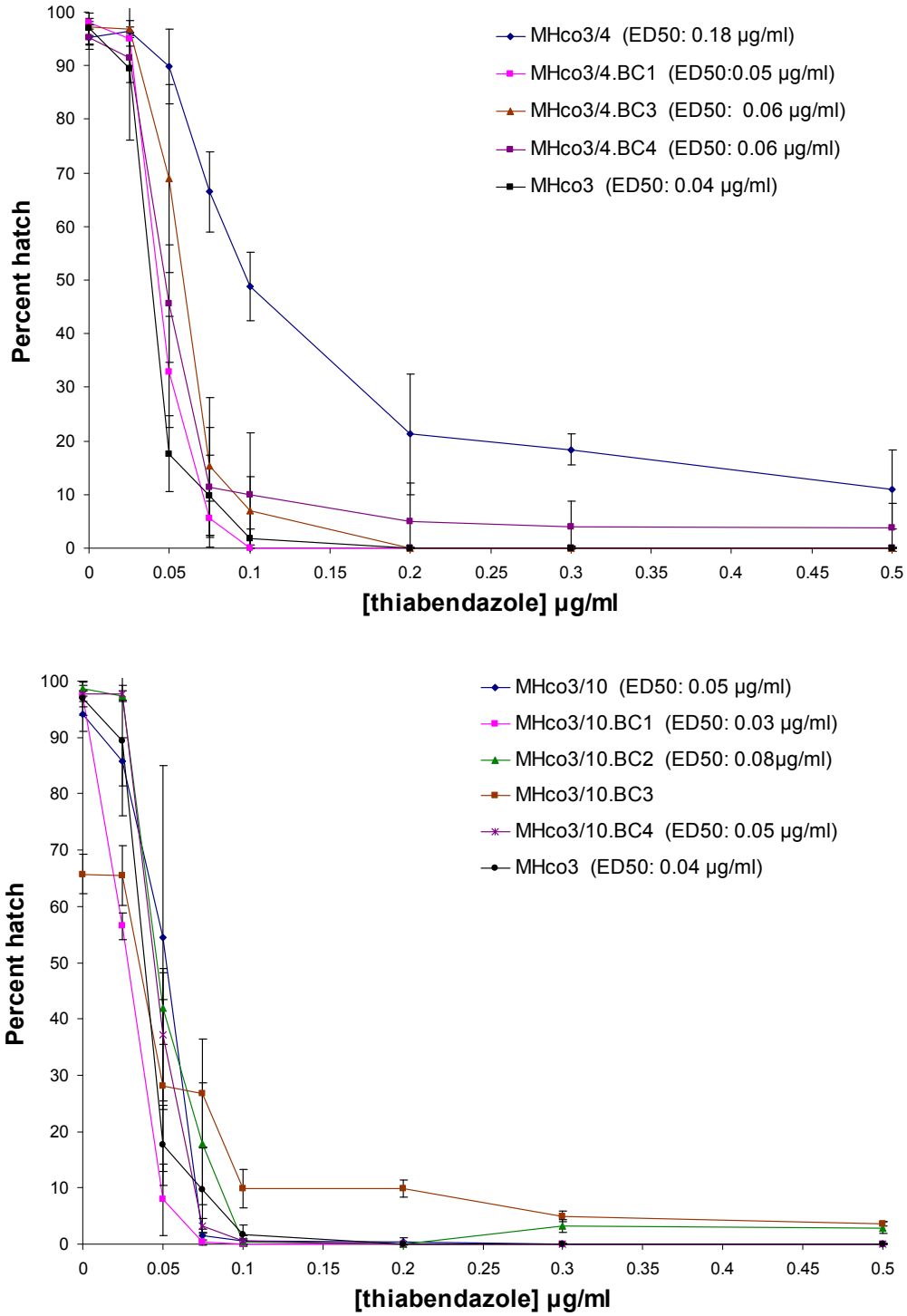


Fig 5.4: Dose response curves generated by egg hatch assays using progeny of the first genetic cross and subsequent backcrosses. Error bars show standard deviations.

The mean ED_{50} values (± standard deviations) generated for the progeny of the first genetic cross and subsequent backcrosses are shown in Fig 5.5. The value generated for the F_1 progeny of the MHco3/4 genetic cross is higher than the 0.1 µg/ml thiabendazole threshold that is used to define benzimidazole resistance in the egg hatch assay. ED_{50} values for subsequent backcross generations were lower demonstrating benzimidazole susceptible phenotypes. The values generated for the MHco3/10 genetic cross and for each backcross are lower than the 0.1 µg/ml thiabendazole threshold, indicating benzimidazole susceptibility.

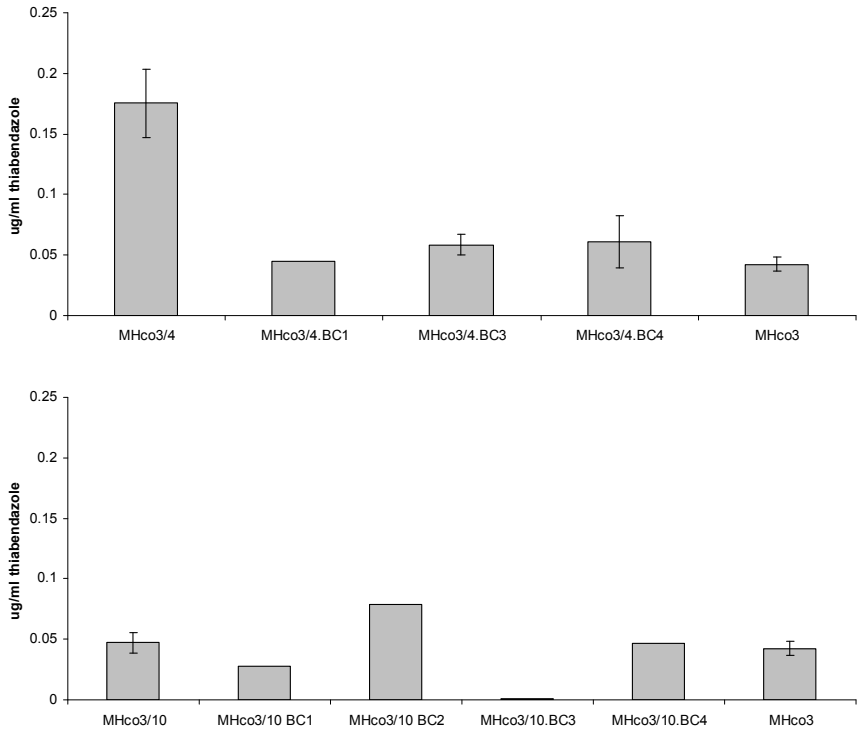


Fig 5.5: Mean (±SD) ED_{50} values generated for the progeny of the first genetic cross and subsequent backcrosses.

5.3.2.2 Larval feeding inhibition assays

Larval feeding inhibition assays were performed on two or three separate occasions on the progeny of each genetic cross and backcross (with the exception of MHco3/4.BC_2 and MHco3/10.BC_3) and on five occasions on L_1 hatched from eggs recovered from

contemporary MHco3 (ISE) donor sheep. Assays could not be performed on MHco3/4.BC_2 and MHco3/10.BC_3 L_1, because the FWECs of the recipient sheep were too low to yield sufficient L_1.

The dose response curves produced for the L_1 progeny of the first genetic crosses differed slightly at higher drug concentrations from those produced using from L_1 from contemporary MHco3 *H. contortus* donors (labelled as MHco3(A) in Figs 6.6 and 6.7), that were performed at the same time. The profiles of the dose response curves in the progeny of the first genetic crosses were more resistant to *in vitro* ivermectin exposure than those of the ivermectin susceptible MHco3 (ISE) strain of *H. contortus*. However, the standard deviations were large and the dose response curves generated for the MHco3/10 progeny were influenced by poor feeding in negative control assays. The dose response curves produced for the L_1 progeny of each backcross were similar to those produced using from L_1 from contemporary MHco3 (ISE) *H. contortus* donors (labelled as MHco3(B) in Figs 6.6 and 6.7), that were performed at the same time. Dose response curves, based on the mean percentage of fed L_1 in each assay (± standard deviations) are shown in Fig 5.6. The mean LFI_{90} and LFI_{99} values (± standard deviations) generated for the progeny of the first genetic cross and subsequent backcrosses are shown in Fig 5.7.

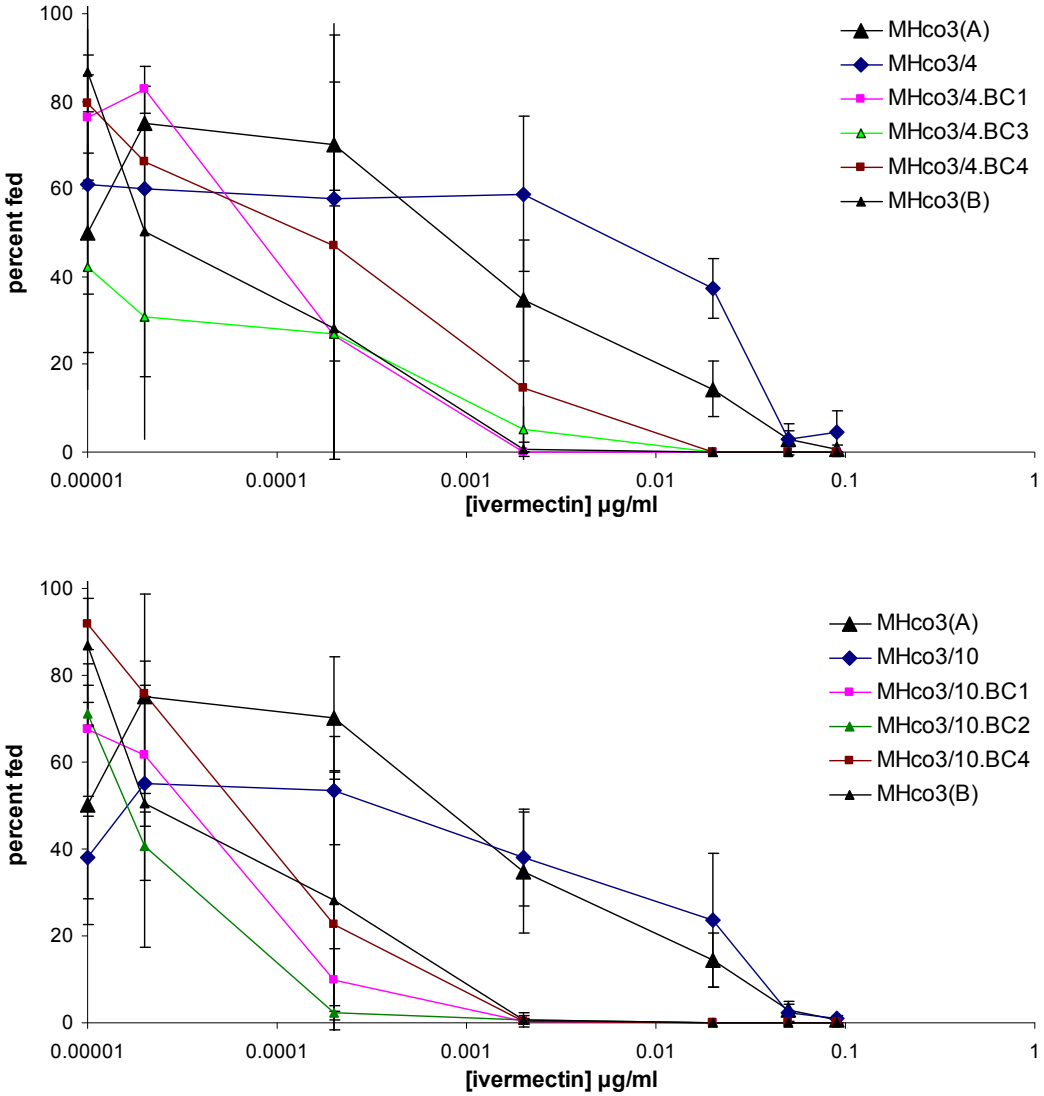


Fig 5.6: Dose response curves generated by larval feeding inhibition assays using progeny of the first genetic cross and subsequent backcrosses. Error bars show standard deviations.

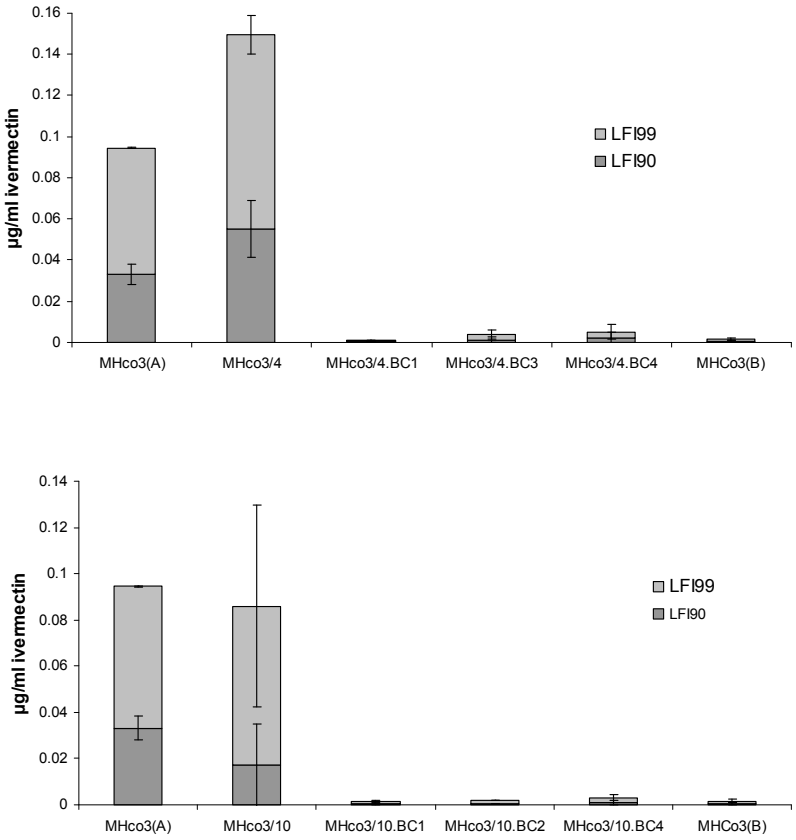


Fig 5.7: LFI_{90} and LFI_{99} (±SD) values generated for the progeny of the first genetic cross and subsequent backcrosses.

5.3.3 Molecular genetic evidence of the success of the backcrosses

5.3.3.1 Bulk microsatellite genetic fingerprints

First the presence or absence of alleles was noted in the genetic cross F_1 and different backcross generations. Table 5.2 shows the discernable alleles that were present in the replicated pooled and bulk preparations of the MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) parent populations, progeny of the first genetic cross and progeny of the backcrosses. Different alleles were present at 9 microsatellite loci between the MHco3 (ISE) and MHco4 (WRS) strains and at 16 loci between the MHco3 (ISE) and

MHco10 (CAVR) strains. Some (but not all) alleles of marker Hcms8a20 derived from the ivermectin resistant MHco4 (WRS) and MHco10 (CAVR) parent populations were present in the progeny of the first genetic cross and of each backcross.

Overall the pattern of presence and absence of strain-specific alleles was very consistent with successful backcrossing. Those alleles specific to the MHco4(WRS) and MHc010(CAVR) strains of *H. contortus* disappeared from the backcross progeny after 2 or 3 generations. In contrast, those specific to the MHco3(ISE) strain of *H. contortus* were retained through to the fourth backcross. Only two markers provided anomalous results in this respect. Alleles of marker HcmsX256 derived from MHco10 (CAVR) parent population were present in the MHco3/10, MHco3/10.BC_1 MHco3/10.BC_2 MHco3/10.BC_3 and MHco3/10.BC_4 progeny, but alleles of the same marker derived from MHco4 (WRS) parent population, were absent from the MHco3/4.BC_2, MHco3/4.BC_3 and MHco3/4.BC_4 progeny. Alleles of marker 8a20 derived from both ivermectin resistant parent populations were present in all of the backcross progeny populations. For the remainder of the microsatellite markers, the MHco3/4 and MHco3/4.BC_1 populations contained alleles derived from both the MHco3 (ISE) and MHco4 (WRS) parent populations, while the MHco3/4.BC_2, MHco3/4.BC_3 and MHco3/4.BC_4 populations only contained alleles derived from the parent MHco3 (ISE) parent population. The MHco3/10 and MHco3/10.BC_1 populations contained alleles derived from both the MHco3 (ISE) and MHco10 (CAVR) parent populations. Alleles derived from the parent MHco10 (CAVR) population were present in the MHco3/10.BC_2 population at 5 microsatellite loci (Hcms36; HcmsX142; HcmsX146; HcmsX151; and HcmsX182), but were absent from the MHco3/10.BC_3 and MHco3/10.BC_4 populations.

Microsatellite allele frequencies of pooled or bulk lysates made from populations of *H. contortus* were estimated from their relative peak heights on the Genescan traces. This process enabled the comparison of genetic fingerprints at microsatellite loci, where the same alleles were present in the parental populations, but at different frequencies. For

example, based on the Genescan peak heights, allele 148 at the Hcms36 microsatellite locus is present in about 50% of the MHco3 (ISE) parental population and about 10% of the MHco4 (WRS) parental population. The 148 allele frequency estimated from the relative peak heights on Genescan traces for MHco3/4 F_1 populations is about 30%, which is an intermediate frequency between those found in the parental populations as predicted for F_1 progeny of a successful genetic cross between the two populations. The allele 148 frequencies of the subsequent backcross populations are similar to those of the MHco3 (ISE) parental population (Fig 5.8).

The estimated microsatellite allele frequencies in the F_1 progeny of the first genetic crosses were also as predicted for other more polymorphic loci. For example, based on the Genescan peak heights for the 3561 microsatellite locus, alleles 261 and 285 are present in about 60% and 40% respectively of the MHco3 (ISE) parental population, while alleles 261, 278, 282, 289 and 292 are present in about 40%, 20%, 10%, 30% and 10% respectively of the MHco10 (CAVR) parental population. The predicted frequencies of alleles 261, 278, 282, 285, 289 and 292 in a genetic cross between these populations would be 50%, 10%, 5%, 20%, 15% and 5% respectively, which is in approximate agreement with the allele frequencies estimated from the Genescan peak heights (Fig 5.9). Again after 2 or 3 backcross generations, the allele frequencies resembled those of the parental MHco3(ISE) population.

The allele frequencies of 17 of 19 microsatellite loci in both third and fourth backcross populations, estimated from their relative peak heights on the Genescan traces, were similar to those of the parent MHco3 (ISE) *H. contortus* population (for example, Figs 6.8 and 6.9). There were just two loci that were anomalous in this respect. The allele frequencies of microsatellite locus Hcms8a20 in both third and fourth backcross populations, estimated from their relative peak heights on the Genescan traces, differed from those of the parent MHco3 (ISE) *H. contortus* population (Fig 5.10). Alleles 244 and 248, that are not present in MHco3 (ISE) populations were retained in the MHco3/10.BC_4 and MHco3/4.BC_4 populations respectively.

The X chromosome microsatellite marker (HcmsX256) allele 243, derived from the MHco10 (CAVR) strain of *H. contortus*, persisted in the MHco3/10.BC_3 and MHco3/10.BC_4 populations. Corresponding persistence of a MHco4 (WRS) strain of *H. contortus* allele in the MHco3/4.BC_3 and MHco3/4.BC_4 populations was not seen (Fig 5.11).

Examples of the Genescan traces for each microsatellite marker are shown in Appendices 5.1 and 5.2.

Marker	MHco3	MHco4	MHco3/4	MHco3/4.BC$_1$	MHco3/4.BC$_2$	MHco3/4.BC$_3$	MHco3/4.BC$_4$
8a20	192; 196; 232; 236; 240	192; 196; 236; 240; 244; 248	192; 196; 232; 236; 240; 244; 248	192; 196; 232; 236; 240; 244; 248	192; 196; 232; 236; 240; 244; 248	192; 196; 232; 236; 240; 244; 248	192; 196; 232; 236; 240; 244
22co3	234; 250	234; 242; 250; 258	234; 250	234; 250	250	234; 250	234; 250
3561	261; 285	261; 285	261; 285	261; 285	261; 285	261; 285	261; 285
18210	219: 225	219: 225	219: 225	219	219: 225	219: 225	219: 225
26981	293	293	293	293	293	293	293
40506	340; 344; 348	340; 344; 348	340; 344; 348	340; 344; 348	340; 344; 348	340; 344; 348	340; 344; 348
60435						114	114
181881	247	247; 251	247; 251	247; 251	247	247	247
Hcms25	209; 211; 213; 215; 217	205; 207; 209; 211; 213; 215; 217	207; 209; 211; 213; 215; 217	209; 211; 213	209; 211	209; 211; 213; 215; 217	209; 211; 213; 215; 217
Hcms27	358	358	358	358	358	358	358
Hcms33	204; 218	204; 218	204; 218	204; 218	204; 218	204; 218	204; 218
Hcms36	148; 152	148; 152	148; 152	148; 152	148; 152	148; 152	148; 152
Hcms40	285; 297	295; 297	285; 295; 297	285; 297	285; 297	285; 297	285; 297
HcmsX142	177, 178; 179; 180	174; 175: 178; 179; 180	175; 178; 179; 180	175; 178; 179; 180	178; 179	178; 179	178; 179; 180
HcmsX146	147: 151	149; 151; 155	147; 149: 151	147; 149; 151; 155	147: 151	147: 151	147: 151
HcmsX151	226; 234	226; 234	226; 234	226; 234	226; 234	226; 234	226; 234
HcmsX182	372; 374	372	372; 374	372; 374	372; 374	372; 374	372; 374
HcmsX256	239; 241; 245	239; 241; 243	239; 241; 243; 245	239; 241; 243; 245	239; 241; 245	239; 241; 245	239; 241; 245
HcmsX337	331; 339	331; 339	331; 339	331; 339	331; 339	331; 339	331; 339

Marker	MHco3	MHco10	MHco3/10	MHco3/10.BC$_1$	MHco3/10.BC$_2$	MHco3/10.BC$_3$	MHco3/10.BC$_4$
8a20	192; 196; 232; 236; 240	204; 208; 240; 244; 248	192; 196; 232; 236; 240; 244; 248	192; 196; 232; 236; 240; 244; 248	192; 196; 232; 236; 240; 244; 248	192; 196; 232; 236; 240; 244; 248	192; 196; 232; 236; 240; 244; 248
22co3	234; 250	250; 258	250	250	234; 250	234; 250	234; 250
3561	261; 285	261; 273; 282; 289; 292	261; 273; 282; 285; 289; 292	261; 282; 285; 289; 292	261; 285	261; 285	261; 285
18210	219: 225	219	219: 225	219: 225	219: 225	219: 225	219: 225
26981	293	254; 282; 293	254; 293	254; 293	293		
40506	340; 344; 348	336: 340		340; 344	340; 344; 348	340; 344; 348	340; 344; 348
60435						107	
181881	247	247; 251	247; 251	247	247	247	247
Hcms25	209; 211; 213; 215; 217	211; 213		209; 211; 213; 215; 217	209; 211, 213	209; 211, 213	209; 211, 213
Hcms27	358	358	358	358	358	358	358
Hcms33	204; 218	204	204; 218	204; 218	204; 218	204; 218	204; 218
Hcms36	148; 152	148; 150; 152; 154	148; 150; 152	148; 150; 152; 154	148; 150; 152	148; 152	148; 152
Hcms40	285; 297	285; 297	285; 297	285; 297	285; 297	285; 297	285; 297
HcmsX142	177, 178; 179; 180	174; 175: 178; 179; 180	175; 178; 179; 180	175; 178; 179; 180	175; 178; 179; 180	178; 179; 180	178; 179; 180
HcmsX146	147: 151	147; 149; 151	147; 149: 151	147; 149; 151	147; 149; 151	147: 151	147: 151
HcmsX151	226; 234	226; 228	226; 228	226; 234	226; 228; 234	226; 234	226; 234
HcmsX182	372; 374	346; 372	346; 372; 374	346; 372	346; 372; 374	372; 374	372; 374
HcmsX256	239; 241; 245	239; 243	239; 241; 243; 245	239; 241; 243; 245	239; 241; 243; 245	239; 241; 243; 245	239; 241; 243; 245
HcmsX337	331; 339	329; 331; 344	329; 331; 339; 344	331	331; 339	331; 339	331; 339

Table 5.2: Discernable alleles in the pooled and bulk preparations of the MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) parent populations, progeny of the first genetic cross and progeny of the backcrosses. (Alleles only present in the parent MHco3 (ISE) population are highlighted in yellow and alleles only present in the parent MHco4 (WRS) or MHco10 (CAVR) populations are highlighted in green.)

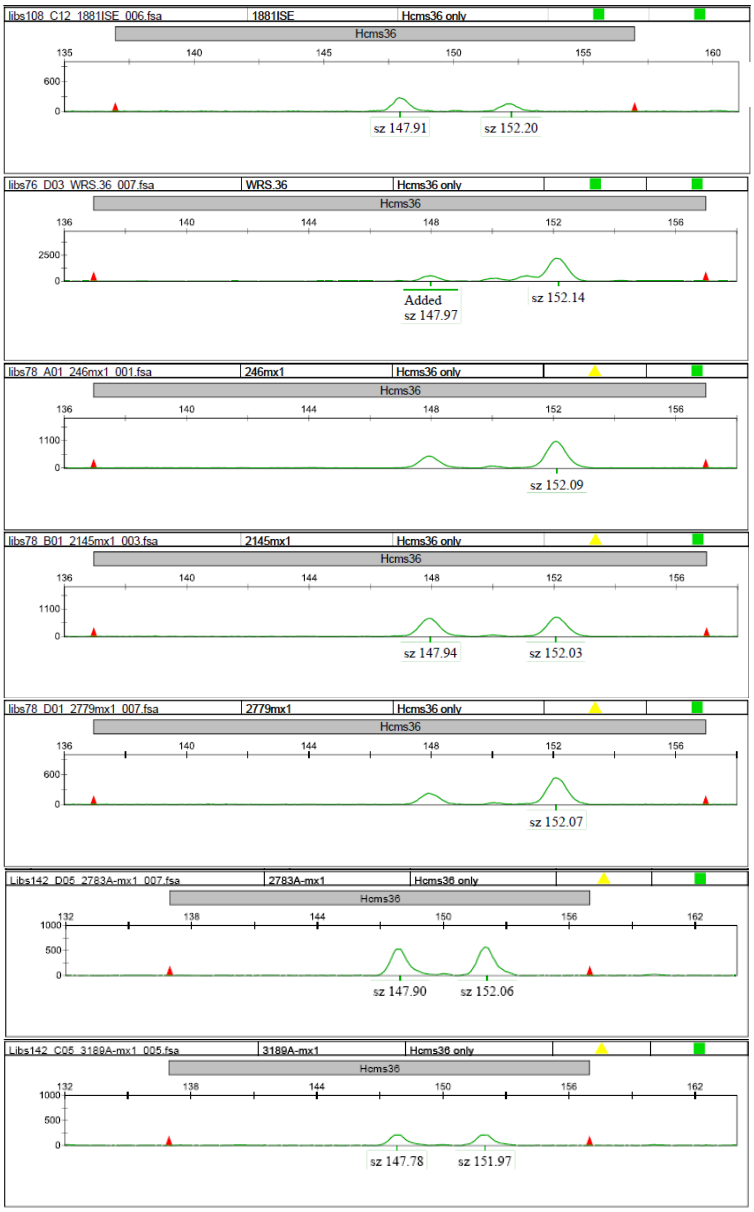


Fig 5.8: Examples of microsatellite marker Hcms36 Genescan traces for MHco3 (ISE) and MHco4 (WRS) parental populations, the genetic cross between the two and subsequent backcrosses.

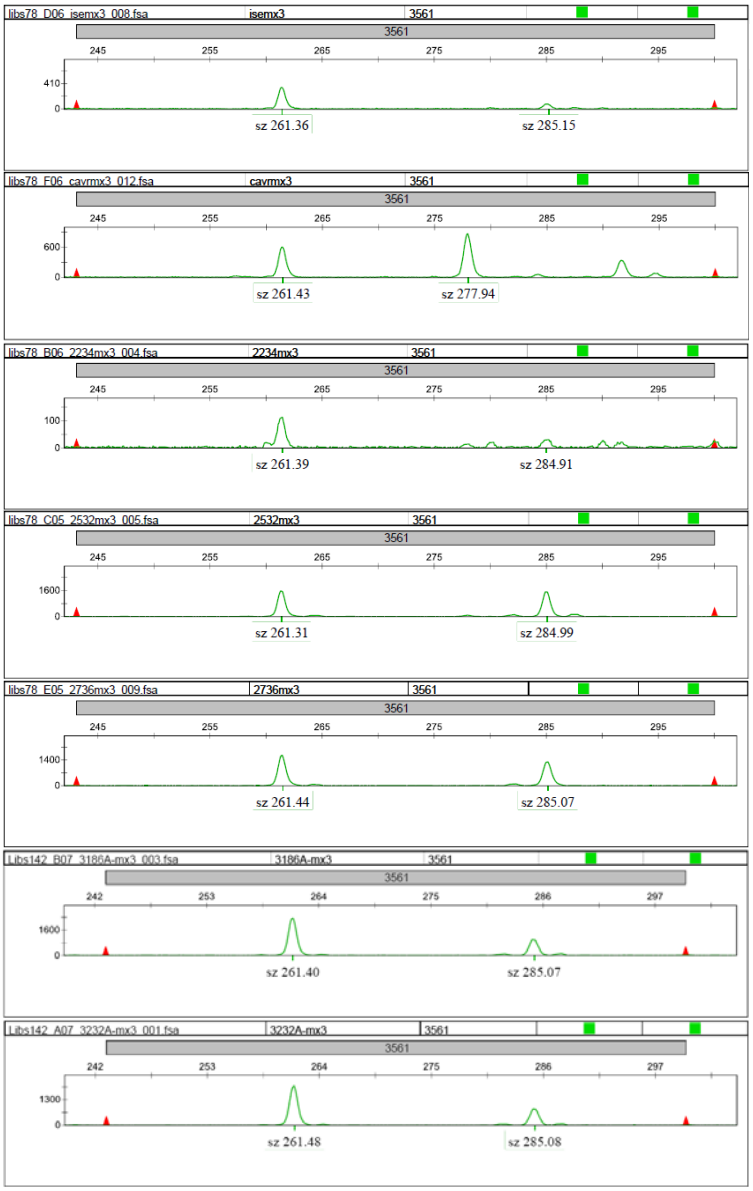
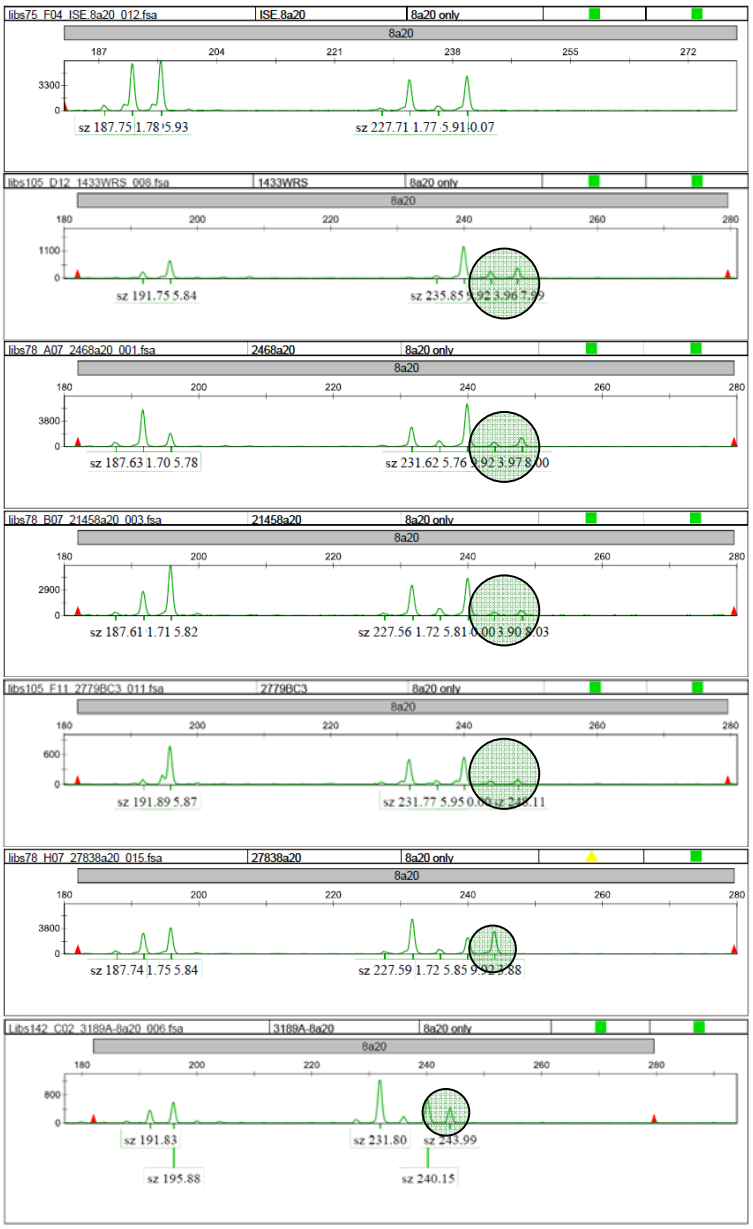


Fig 5.9: Examples of microsatellite marker 3561 Genescan traces for MHco3 (ISE), MHco10 (CAVR), MHco3/10 and MHco3/10.BC_4 populations. (The allele frequencies described in the text are derived form the mean of replicates for each population, while only individual examples are shown.)



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

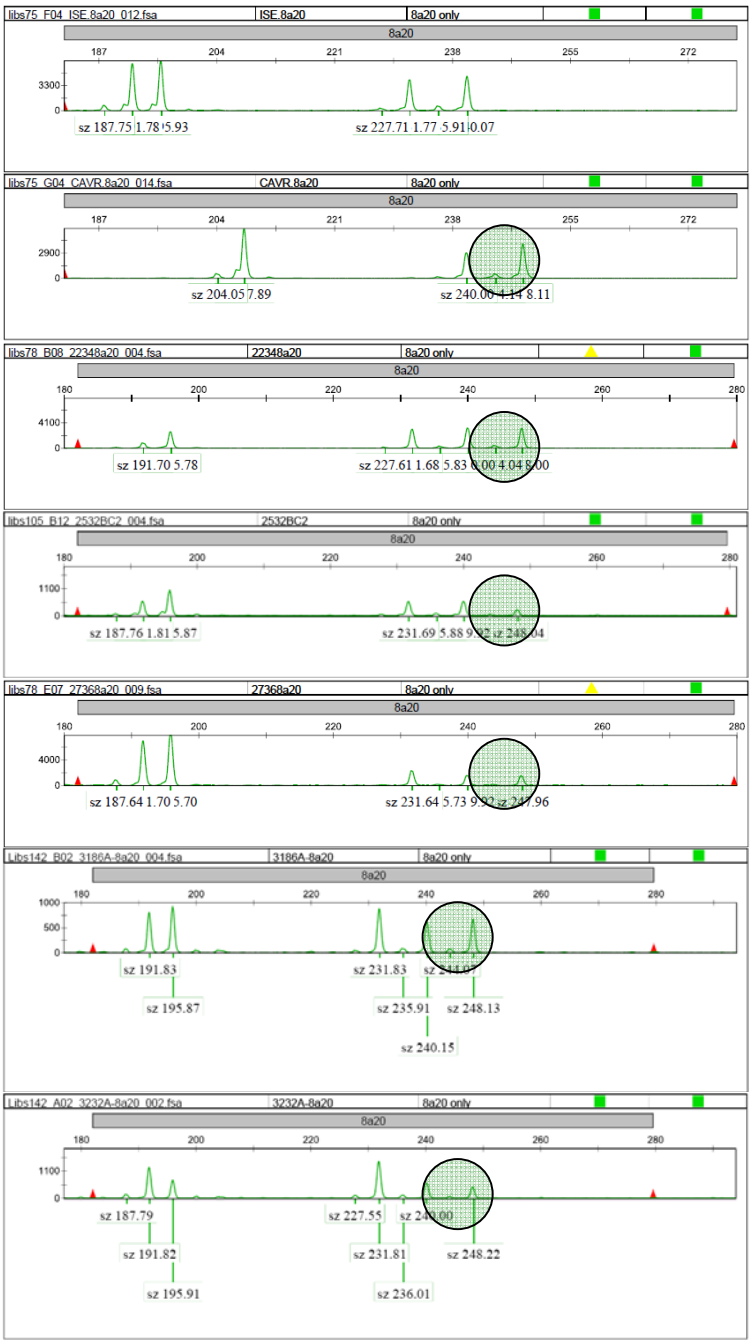
MHco3/4.BC$_1$

MHco3/4.BC$_2$

MHco3/4.BC$_3$

MHco3/4.BC$_4$

Fig 5.10



MHco3 (ISE)

MHco10 (CAVR)

MHco3/10

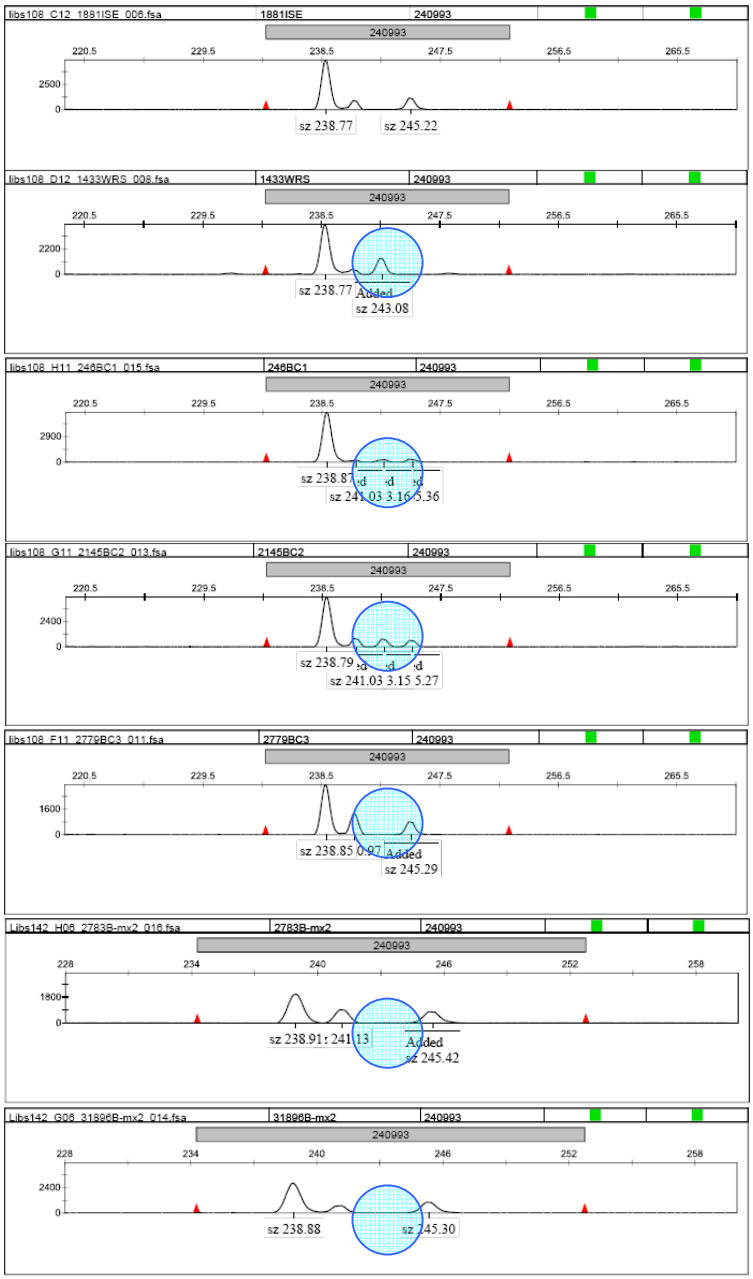
MHco3/10.BC$_1$

MHco3/10.BC$_2$

MHco3/10.BC$_3$

MHco3/10.BC$_4$

Fig 5.10: Genescan traces for microsatellite marker 8a20. Alleles 244 and 248, that are not present in MHco3 (ISE) populations are clearly present in the MHco3/10.BC$_4$ and MHco3/4.BC$_4$ populations respectively.



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

MHco3/4.BC_1

MHco3/4.BC_2

MHco3/4.BC_3

MHco3/4.BC_4

Fig 5.11

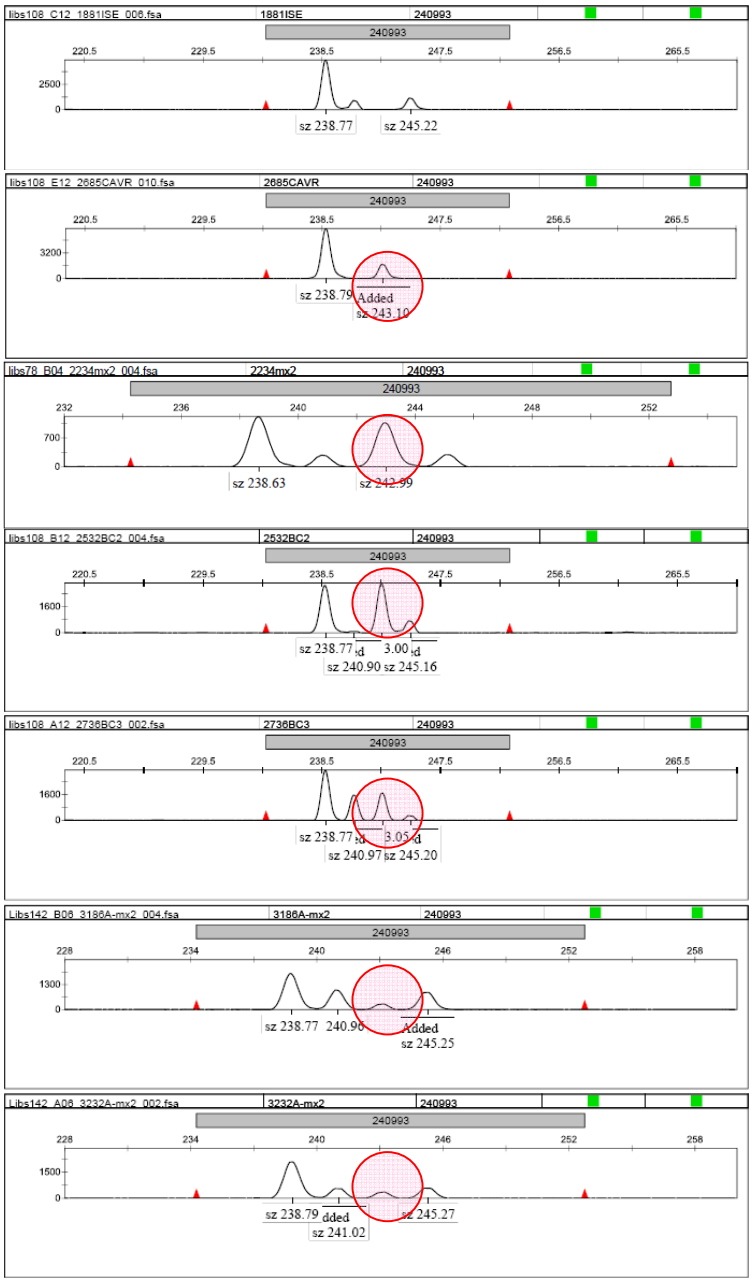


Fig 5.11: Genescan traces for microsatellite marker HcmsX256, showing the presence of allele 243 in the MHco3/10.BC_4 population.

5.3.3.2 Efficacy of ivermectin

5.3.3.2.1 MHco3(ISE)

No *H. contortus* were recovered from the abomasa of the two MHco3 (ISE) donor sheep 10 days after oral treatment with a half therapeutic dose of 100 μg/kg of ivermectin on days 11 and 21 after infection with 10,000 or 5,000 MHco3 (ISE) L_3 respectively. A 100% reduction in FWEC was shown in the donor sheep infected on day 21. These results show that both mature adults and day 11 L_4s of the parental ivermectin susceptible MHco3 (ISE) strain of *H. contortus* are susceptible to a half therapeutic dose of 100 μg/kg of ivermectin.

5.3.3.2.2 MHco3/10.BC_2

Oral treatment of a MHco3/10.BC_2 donor sheep with a half therapeutic dose of 100 μg/kg of ivermectin led to a 77% reduction in FWEC, 12 days after treatment. About 3000 *H. contortus* were recovered from the abomasum when the donor sheep was killed one month after treatment, indicating that the treatment was not wholly successful.

5.3.3.2.3 MHco3/4.BC_4 and MHco3/10.BC_4

Oral treatment of MHco3/4.BC_4 and MHco3/10.BC_4 donor lambs with a full therapeutic dose of 200 μg/kg of ivermectin led to 95.9% and 99.8% reductions in FWECs respectively, 12 days after treatment.

5.3.4 Archiving of material

Material has been archived as a resource for future genomic and molecular studies as shown in Table 5.3.

Sheep/sample ID	Isolate	Bulk lysates	LateL4/adults in ethanol	Cryporeserved live L3	10oC live L3	FWEC data	EHA data	LFIA data	Microsatellite data
926D	MHco3	✓	✓						✓
966D	MHco4	✓	✓						✓
967D	MHco10	✓	✓						✓
246D	MHco3/4	✓	✓		✓	✓	✓	✓	✓
374D	MHco3/10	✓	✓			✓	✓	✓	✓
2710E	MHco3		✓						
2240E	MHco3/4		✓						
2234E	MHco3/10		✓						
2145E	MHco3/4.BC1	✓	✓	✓	✓	✓	✓	✓	✓
2532E	MHco3/10.BC1	✓	✓	✓	✓	✓	✓	✓	✓
2680E	MHco3		✓						
2768E	MHco3/4.BC1		✓						
2357E	MHco3/10.BC1		✓						
2779E	MHco3/4.BC2	✓	✓	✓	✓	✓			✓
2736E	MHco3/10.BC2	✓	✓	✓	✓	✓	✓	✓	✓
2551E	MHco3		✓						
2725E	MHco3/4.BC2		✓						
2783E	MHco3/4.BC3	✓	✓	✓	✓	✓	✓	✓	✓
3188E	MHco3		✓						
3194E	MHco3/4.BC3		✓						
3199E	MHco3/10.BC2		✓						
3189E	MHco3/4.BC4	✓		✓	✓	✓	✓	✓	✓
3186E	MHco3/10.BC3	✓	✓	✓	✓	✓	✓		
3213E	MHco3		✓						
3103E	MHco3/10.BC3		✓						
3232E	MHco3/10.BC4	✓		✓	✓	✓	✓	✓	✓

Table 5.3: Genetic material derived from the backcrossing experiments that is available for future use and information presented in this thesis to characterise isolates.

5.4 Discussion

H. contortus is one of the few parasitic nematode species in which genetic backcrosses between strains have been successfully undertaken. The aims of these genetic backcrosses have been: to determine the dominance of benzimidazole resistance and to demonstrate sex linkage through different reciprocal crosses between male and female nematodes (Le Jambre and others, 1979); to study reproductive abnormalities arising from hybridisation studies between *H. contortus* and *H. placei* (Le Jambre and Royal, 1980); and to study the involvement of candidate P-glycoprotein genes with macrocyclic lactone resistance (Le Jambre and others, 1999b). The latter study involved first performing a genetic hybridisation cross between a population of day 14 sexually immature female macrocyclic lactone resistant CAVR *H. contortus* and a similarly aged population of male *H. placei*, then backcrossing ivermectin surviving F_1 hybrid generation females with *H. placei* males. The BC_1 progeny was then treated with ivermectin, allowing resistance genes derived from the parental *H. contortus* population to be retained in the hybrid backcross population having predominantly *H. placei* DNA. Candidate genes were then compared between the backcross population and the original CAVR *H. contortus*.

The aim of the current genetic backcrossing method was to create nematode populations with genomes that are essentially those of the anthelmintic susceptible MHco3 (ISE) strain that has been used for the *H. contortus* genome project, embedded in which are anthelmintic resistance loci and linked DNA derived from the field selected MHco4 (WRS) and genetically divergent MHco10 (CAVR) strains of *H. contortus*. Any polymorphisms then identified by genomic comparisons between the parental MHco3 (ISE) strain and the backcross populations of *H. contortus* could be linked to the genes conferring anthelmintic resistance. This genetic backcrossing method differed from the previous methods (Le Jambre and others, 1979; Le Jambre and Royal, 1980; Le Jambre and others 1999b) because the backcross was repeated several times in order to remove the background DNA derived from the resistant *H. contortus* populations. The method

itself proved to be straightforward and robust, because it involved crosses between populations of nematodes rather than single parents. Support for the success of the genetic backcross was sought by comparison of the Genescan traces for a panel of microsatellite markers between the MHco3 (ISE) strain and the MHco3/4.BC_4 and MHco3/10.BC_{10} populations of *H. contortus*, and by the demonstration of phenotypic resistance to ivermectin when the backcross populations were exposed to treatment of their sheep host with a half therapeutic dose of 100 μg/kg.

Comparison of the bulk microsatellite Genescan traces showed that the MHco3/4.BC_4 and MHco3/10.BC_{10} populations were indistinguishable at 17 of 19 loci from the MHco3 (ISE) strain of *H. contortus*, while an indeterminable proportion of the first genetic cross and the first three backcross generations survived treatment of donor lambs with a half dose of 100 μg/kg of ivermectin, 11 days after infection with L_3. By comparison, the MHco3 (ISE) *H. contortus* were shown to be susceptible to a half dose of 100 μg/kg of ivermectin, even when it was administered 11 days after infection of the donor lamb. Hence the genetic and phenotypic evidence are consistent with the expectations of a successful backcross (phenotypes consistent with selection for resistance to a half therapeutic dose of 100μg/kg of ivermectin, with a largely MHco3(ISE) genetic background).

It is possible that the identification of alleles derived from the ivermectin resistant parental MHco4 (WRS) and MHco10 (CAVR) strains in the MHco3/4.BC_4 and MHco3/10.BC_4 populations of *H. contortus* at the Hcms8a20 microsatellite locus was due to linkage to a gene conferring resistance. However, the parental MHco4 resistant population contained two alleles that were not present in the MHco3 (ISE) strain of *H. contortus*, while the fourth backcross populations only differed in one allele. If the microsatellite locus was linked to a gene conferring resistance, then each of the alleles derived from the parental resistant population might be expected to be represented proportionally in the fourth backcross population, depending on the haplotype background of the resistant polymorphism in the parental populations. While the

Hcms8a20 microsatellite Genescan traces for the MHco3/4.BC_4 and MHco3/10.BC_4 populations of *H. contortus* could be evidence of linkage to the resistance phenotype, they could also have resulted from genetic drift or PCR related artefacts. This highlights the requirement for a much larger panel of more than 100 reliable polymorphic microsatellite markers, distributed throughout the genome and located on a genetic or physical map. If enough mapped markers are used to anaylse the backcrosses, then regions of the genome genuinely linked to resistance-conferring polymorphism would be indicated by the retention of alleles specific to the resistant parental strain in several genetically linked loci.

The persistence of an allele of the HcmsX256 X chromosome microsatellite locus derived from ivermectin resistant parental MHco10 (CAVR) strain of *H. contortus* in the MHco3/10.BC_4 population could imply a sex-linked mechanism of resistance. Furthermore the absence of persistence of a corresponding allele in the MHco3/4.BC_4 population could imply a strain specific ivermectin resistance mechanism. This would be consistent with the results of previous studies that have shown macrocyclic lactone resistance in the Hco10 (CAVR) strain of *H. contortus* to be independent of resistance to other anthelmintic groups (Gill and others, 1995) and inherited as a dominant trait (Le Jambre, 1993). Adult males appear to be more sensitive to ivermectin than adult females, which may be due to the physiology and behaviour of the males, but may also imply a sex-linked inheritance of resistance (Le Jambre and others, 1995).

Preliminary studies described in chapter 3 showed that the post ivermectin treatment efficacies for the F_1 progeny of a genetic cross between the ivermectin susceptible MHco3 (ISE) and ivermectin resistant MHco4 (WRS) strains of *H. contortus* were 98% following the use of a full therapeutic dose rate of 200 μg/ml of ivermectin and 0% following the use of a half therapeutic dose. The half therapeutic dose rate (Palmer and others, 2001) was therefore chosen for the first genetic cross and subsequent backcrosses, to ensure that *H. contortus* that had survived anthelmintic treatment could be recovered from donor sheep for surgical transfer. However, it is acknowledged that

this regime may have selected for different resistance mechanisms when compared to full dose selection. The preliminary FECRTs that were conducted by treating single donor lambs 29 days after infection with MHco3/4.BC_4 or MHco3/10.BC_4 *H. contortus* L_3 with 200 μg/ml ivermectin, showed post treatment efficacies of 95.9% and 99.8% respectively. These results confirm the survival of ivermectin resistant nematodes, but are greater than the post anthelmintic treatment efficacy threshold of 95% (Coles and others, 1992; Coles and others, 2006) that is used to define resistance. Selection with a half therapeutic dose of ivermectin may therefore have only selected for resistance to a full therapeutic dose in a small number of nematodes, implying the involvement of different additional resistance mechanisms. However, post treatment efficacies based on the FWECs of individual sheep are uninformative, due to variation that exists between animals in drug pharmacokinetics and in their immune response to parasitic nematode infection. Furthermore, the FECRTs were performed in September using 6 month-old lambs, which could have acquired non-specific immunity that may have re-enforced the effects of ivermectin treatment (Hennessy, 1994). Similar circumstances have been identified in field infections, whereby the FECRT has failed to diagnose the known presence of ivermectin resistance in 6 month-old Mule lambs (Sargison and others, 2007a). The *in vivo* anthelmintic resistance phenotype of the MHco3/4.BC_4 or MHco3/10.BC_4 *H. contortus* can most reliably be determined using a critical efficacy test. This would also enable the contemporary characterisation of the *in vitro* anthelmintic resistance phenotype. This experiment was not possible within the time frame of the thesis because it needs to be conducted in young lambs. A detailed critical efficacy test of the backcross strains is planned as soon as appropriate numbers of young lambs are available, in order to define resistance phenotype of the backcross lines in more detail.

The *in vitro* bioassays helped to characterise the two backcross lines and again the results were consistent with the results anticipated from successful backcrosses. Predictably there were no differences in the EHA between the MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus*, the F_1 progeny of the MHco3/10 genetic cross

and the subsequent backcross populations, all being susceptible to thiabendazole in the EHA. The F_1 progeny of the MHco3/4 genetic cross between populations of benzimidazole susceptible MHco3 (ISE) and resistant MHco4 (WRS) strains of *H. contortus* was resistant to thiabendazole in the EHA, while the subsequent backcrosses were indistinguishable from the benzimidazole susceptible MHco3 (ISE) parental strain of *H. contortus*. The results for the first genetic cross imply at least partial dominance of the benzimidazole resistance phenotype in the EHA, but would have been influenced by genetic diversity in both parental strains. Benzimidazole susceptibility in the subsequent backcross populations, while the first genetic cross was resistant, is as expected since selection was not applied for benzimidazole during the backcrossing process. This result also suggests that separate mechanisms confer resistance to thiabendazole in the EHA, and resistance to a half dose of ivermectin *in vivo*, and that these mechanisms are not genetically linked.

The LFIAs were uninformative and did not demonstrate any significant differences in the ivermectin resistance phenotype. The results are probably unreliable because the assays for each generation were performed at different times of year and in different aged lambs. The *in vitro* bioassays also could not be used to lend support to the success of the backcrosses, because of the potential involvement of resistance mechanisms that may not have been selected for *in vivo* using the half therapeutic dose of 100 µg/kg of ivermectin.

The MHco3/4.BC_4 and MHco3/10.BC_4 populations now provide a valuable resource for future genetic and genomic studies of the basis of ivermectin resistance in two commonly used laboratory strains of *H. contortus*. This resource has been protected by cryopreservation of L_3 and by the further establishment of the MHco3/4.BC_4 and MHco3/10.BC_4 *H. contortus* populations in donor lambs. The key feature of these lines is that their genetic background is predominantly that of MHco3(ISE) *H. contortus*, the ivermectin susceptible strain used for the genome sequencing project, into which resistance loci from two different ivermectin resistant strains have been introgressed.

The MHc04 (WRS) strain, although passaged for many years in the laboratory, was originally isolated as an ivermectin resistant parasite population from the field, as opposed to being artificially selected in the laboratory. Hence there is a reasonable expectation that the key resistance genes in this strain will be relevant to those of importance in the field. The origin of the MHco10 (CAVR) strain of *H. contortus* is less clear, but the MHco3/10 backcross lines are nevertheless useful as proof of concept, due its extreme genetic divergence from the MHco3 (ISE) strain.

There are a number of ways the backcross strains can be utilised. Firstly, they will provide a resource for genetic mapping of the loci conferring resistance to a half therapeutic dose of 100µg/kg ivermectin. In order to progress this approach a larger panel of polymorphic microsatellite markers will be needed. These markers will need to be located on a physical or genetic map so that their relative positions in the genome are known. The markers will also need to be polymorphic and have significant differences in the presence or absence, or frequency of alleles between the parental strains of the backcross. Once such a panel of markers has been developed they can be used to genotype the backcross strains to discern which parts of the genome are derived from either parental strain. Regions of the genome containing resistance loci will be derived from the resistant parental isolates whereas the rest of the genome should be derived from the MHco3(ISE) isolate. Access to a fully assembled annotated genome sequence or at least a genetic map based on several hundred markers is a prerequisite for this analysis. As neither is currently available, the resource created by the genetic backcross is in some regards ahead of its time, however the archiving of the material will allow these approaches to be applied when they become possible.

The ivermectin resistant *H. contortus* backcross populations are also immediately valuable for a variety of molecular biology and genomic approaches to study the molecular basis of ivermectin resistance. For example, comparison of the polymorphisms between the parental and backcross strains will be a powerful approach to investigate the possible association of candidate genes with resistance. Since the

genetic background is predominantly MHco3(ISE), any associations between candidate gene polymorphisms and the resistance phenotype are more likely to be significant than apparent associations between the parental ivermectin resistant and susceptible strains where there is much genetic variation throughout the genome. The ivermectin resistant *H. contortus* backcross populations could also be immediately valuable for whole genome approaches such as differential display, expression microarrays and whole genome tiling arrays to look for associations of genome wide polymorphisms with the ivermectin resistance phenotype. Again any differences between the backcross and susceptible MHco3(ISE) strain are more likely to be significant than comparisons between the MHco3(ISE) and parental resistant strains. For example, if the expression of a particular gene is up-regulated in the backcross strains relative to the MHco3(ISE) strain, it is likely to be in a region of the genome genetically linked to resistance .

Chapter 6: General discussion

6.1 Summary

The primary aim of the experiments described in this thesis was to develop genetic crossing methods that can be used to expedite the identification of molecular markers linked to genes conferring macrocyclic lactone resistance in *H. contortus*. Experiments used in this course of study led to the development of a novel method that enabled the recovery and culture to L_3 of the progeny of matings between a single male and a single female *H. contortus*. The success of this method was supported by genetic fingerprinting of the filial progenies to demonstrate inbreeding and also to show genetic divergence between different inbred lines. This genetic diversity could be exploited in the construction of genetic maps. A novel genetic backcrossing method was developed and used to produce populations of *H. contortus* with a genetic background essentially the same as the ivermectin susceptible MHco3(ISE) strain that has been used to provide DNA template for the *H. contortus* genome project (http://www.sanger.ac.uk/Projects/H_contortus/), except for regions linked to mutations conferring anthelmintic resistance, that were derived from field selected laboratory strains. This will prove to be a valuable resource for both genetic and functional studies of anthelmintic resistance. Experiments described in this thesis have also made a valuable contribution to our understanding of the basic reproductive biology of the model parasitic nematode, *H. contortus*. This improved understanding was useful for the development of genetic crossing methods and will be important in the analysis and interpretation of any future population genetics studies of the parasite, for example to investigate the genetic origin and evolution of anthelmintic resistance.

6.2 Background

The emergence of multiple anthelmintic resistance presents a serious threat to economically sustainable sheep production. Some sheep flocks in the south east of Scotland have been dispersed because the complexities imposed by the economic management of multiple anthelmintic resistant *Teladorsagia circumcincta* have added to a burgeoning list of constraints to sheep production. Multiple anthelmintic resistance has been identified in flocks selling terminal sire ram lambs and shearlings to large numbers of commercial sheep farmers (for example, Sargison and others, 2007a), and epidemiological investigations of the 2001 foot and mouth outbreak (for example, Mansley and others, 2003) highlighted the great extent of sheep movements within the UK. It is therefore probable that parasitic nematodes carrying genes conferring multiple anthelmintic resistance are present in all UK sheep flocks, albeit currently in most at a low frequency within their total nematode populations and probably at levels that are undetectable using current methods.

Various managemental tools have been developed aimed at slowing the emergence of anthelmintic resistant nematodes in sheep flocks while maintaining satisfactory nematode parasite control using strategic suppressive anthelmintic treatments. In particular, strategies such as targeted selective anthelmintic treatments of the animals that make the greatest contribution to parasitic nematode contamination while allowing for the survival and development on pasture of limited numbers of anthelmintic susceptible nematodes shed by untreated animals (for example, Van Wyk and others, 2006) may prove to be useful. However these strategies require a high level of commitment and managemental input and are unproven on commercial sheep farms in the UK. Sensitive diagnostic tests are therefore required to monitor the effectiveness of strategies aimed at slowing the emergence of anthelmintic resistance and to elucidate the way in which the frequency of resistant nematodes increases on farms, thus enabling the development of new control methods. Likewise, sensitive diagnostic tests will be

required to monitor the efficacy of any new anthelmintic drug group, such as the amino-acetonitrile derivatives (Kaminsky and others, 2008).

PCR to identify molecular markers linked to the mechanism of action of anthelmintic drugs is an obvious candidate for high predictive value diagnostic tests for anthelmintic resistance (for example, Von Samson-Himmelstjerna, 2006). The action of ivermectin is thought to include ligand-gated chloride channels (Dent and others, 2000), but resistance may involve other non-specific drug regulatory mechanisms such as ATP-binding cassette proteins (Blackhall and others, 2008). Most studies to-date aimed at identifying molecular markers for anthelmintic resistance have focused on candidate genes coding for known mechanisms of drug action (for example, Blackhall and others 1998b; Blackhall and others, 2003). In the case of ivermectin resistance such markers have been elusive. Furthermore, even if mutations are identified in candidate genes, provision of conclusive evidence of an association with anthelmintic resistance may be problematic (for example, Gilleard, 2006). An alternative approach that does not depend on pre-conceived ideas about candidate genes for anthelmintic resistance might involve performing genetic crosses between anthelmintic susceptible and anthelmintic resistant parents enabling genomic comparisons between the parents and filial progeny. This approach has been widely used in protozoal parasites including *Trypanosoma brucei* (for example, Tait and others, 2002), *Eimeria tenella* (for example, Shirley and Harvey, 2000), *Palsmodium falciparum* (for example, Su and others, 2007) and *Toxoplasma gondii* (for example, Sibley, 2009) with the aim of producing genetically divergent clones for the creation of genetic maps to allow identification of genes determining important traits. However, unlike sheep nematode parasites, these protozoal parasites undergo both sexual reproduction, enabling genetic crosses between inbred populations derived from cloned lines, and asexual reproduction enabling the further generation of clones for analysis. Furthermore, sexual reproduction in host species such as tsetse flies or mosquitoes is experimentally easier to manipulate than it is in the sheep hosts of nematode parasites.

6.3 Discussion

The research in this study first aimed to develop a method to enable a genetic cross between single male and female anthelmintic susceptible and resistant parasitic nematode parents. Conclusive evidence could not be found to suggest that the surgical transfer of a single male ivermectin susceptible and a single female ivermectin resistant late L_4/immature adult *Haemonchus contortus* to the abomasa of recipient sheep resulted in a successful genetic cross. While the approach might eventually have led to the confirmation of a successful single *H. contortus* parent genetic cross, it was abandoned because it became apparent that the accurate determination of the resistance phenotype of parental and progeny nematodes and of its dominance would be impractical. Such phenotypic characterisation would be required for genomic comparisons between resistant and susceptible nematodes.

H. contortus was chosen as the model parasitic nematode for this study because of the ease of experimental infections and because it is the first ruminant parasitic nematode to be used for a genome sequencing project. *H. contortus* is known to be diploid, dioecious and sexually reproducing. The biotic potential of *H. contortus* is known be high and it has recently been shown that adult female parasites in a population mate with several males (Redman and others, *In Press*), leading to a high degree of genetic diversity in their progeny. This diversity offers the potential of adaptation in the face of adverse conditions, such as exposure to anthelmintics, whereby a small number of parasites with favourable genotypes within a large diverse population can survive. The preliminary attempts to perform a single parent genetic cross highlighted deficiencies in the current understanding of the reproductive biology of endoparasitic nematodes. Subsequent studies provided new evidence to show that adult female *H. contortus* continue to shed eggs indefinitely and that egg development and hatching persists for about 15 days after removal from males, suggesting that the adult females store sperm, although, to date, a storage organ has not been identified. Female *H. contortus* were shown not to shed fertilised eggs following their removal from males on day 14 after L_3 infection.

However, research in this study showed that about half of the unfertilised eggs were heterozygous at one or more microsatellite loci. Diploidy or polyploidy may have arisen due to a form of pre-meiotic endomitosis triggered by failure of spermatozoal penetration of the ova, although further studies using cryopreserved material from this study are required to elucidate the mechanism(s) involved. Some of the unfertilised eggs developed morphologically abnormal gastrulae, within which some degree of cell migration appeared to have occurred. Regardless of the precise mechanisms involved, the identification of ploidy and observation of gastrulation in unfertilised eggs might indicate partial parthenogenetic development. These observations of the biology of *H. contortus*, along with existing knowledge of polyandry and high biotic potential, provide further evidence of the adaptability and evolutionary potential of the endoparasitic nematode.

Molecular markers associated with ivermectin resistance will eventually be identified. If these are to be applied in order to determine the risk factors that enable or slow selection for anthelmintic resistance, knowledge of how freely different populations of parasitic nematodes interbreed will be needed. Principal co-ordinate analyses of microsatellite alleles has shown genetic divergence between the three different laboratory strains of *H. contortus* that were used throughout this study. The three strains of *H. contortus* appeared to freely interbreed in the absence of conspecific partners, but preliminary studies provided some evidence of competition between genetically divergent isolates of the same species in a co-infection, with the MHco3 (ISE) strain outcompeting the MHco10 (CAVR) strain of *H. contortus*. Further studies examining the progeny of mixed strains of *H. contortus* at different times after infection are now being undertaken to determine whether or not this is related to differences in the reproductive biology of the different strains at different stages after donor infection.

The effects of donor sheep age and immune competence on host parasitological aspects of *H. contortus* infection were investigated in an attempt to explain the reasons for the poor success of nematode infections of donor and recipient sheep used for genetic

crossing studies during the winter months. While host immunity did not have any effect on the number of *H. contortus* surviving in the abomasum, it had a profound effect on egg shedding by individual female nematodes. Furthermore, this effect varied between different strains of *H. contortus*, which could influence the population genetics of mixed infections. However, neither the age of the donor sheep, nor corticosteroid immune suppression had any effect on egg hatching or larval feeding at different concentrations of anthelmintic drugs in bioassays in this study. Thus, whether or not egg hatching and larval development during the winter months is related to effects of host immunity on parasitic stages or to extrinsic factors acting on free living stages could not be determined.

Following a decision not to pursue the single *H. contortus* parent genetic crossing approach to identify molecular markers linked to anthelmintic resistance, an alternative genetic crossing approach was developed, backcrossing resistance alleles from a resistant isolate into a known susceptible isolate. Genetic crosses were made between populations of susceptible and resistant strains of *H. contortus*, selecting for phenotypically resistant nematodes in the progeny populations and then backcrossing the resistant progeny with the original susceptible population. The premise for this backcrossing method was that polymorphisms in regions of genomic DNA between the original susceptible parent population and progeny population of the final backcross could be linked to genes conferring resistance. Four backcrosses were performed, selecting for resistance using a half therapeutic dose of ivermectin and incorporating resistance genes from MHco4 (WRS) or MHco10 (CAVR) strains into the susceptible genome project MHco3 (ISE) strain of *H. contortus*. Success of the backcrosses was supported by the demonstration of phenotypic resistance to a half therapeutic dose of ivermectin in the fourth backcross populations, which had similar genetic fingerprints to the parent susceptible strain, based on a panel of microsatellite markers thought to be distributed throughout the genome. Further phenotypic characterisation of the backcross populations is now required, in particular using critical efficacy tests. The two fourth backcross populations are a potentially valuable resource for genomic comparison with

the parental ivermectin susceptible, genome project strain of *H. contortus*, as and when a larger panel of mapped microsatellite markers, or new technologies such as whole genome sequencing of individuals become available and affordable. In the meantime, further selection for anthelmintic resistance in the backcrossed populations may prove to be useful, for example selecting with a full dose of ivermectin.

Availability of an annotated, assembled *H. contortus* genome sequence would expedite the purpose of the ivermectin resistant *H. contortus* backcross populations to identify molecular markers and candidate genes for ivermectin resistance. The major obstacle preventing genome assembly is the high level of genetic polymorphism that is present in the MHco3 strain of *H. contortus* that has been used for the genome project (John Gilleard, *personal communication*). This problem could be overcome by developing a single parent mating method to inbreed MHco3 *H. contortus* nematodes. Such a method was developed involving the surgical transfer of 20 female and one male day 14 *H. contortus* to the abomasum of recipient sheep. The recipient sheep were euthanased about 7 days after surgical transfer and individual female *H. contortus* transferred into an *in vitro* system where they were encouraged to lay eggs that were then cultured to L_3. The success of this method was demonstrated by phenotypic characterisation and by using neutral SNP, microsatellite and SSCP markers to show a significant loss of polymorphism in filial populations compared to the parent population. Genotypically divergent inbred lines were produced, which may prove to be useful for the creation of a genetic map, but could also have applications for comparative genomics to study candidate gene SNPs, for the segregation of different aspects of anthelmintic resistance and for the testing of genetic markers.

A low rate of egg hatching and development was identified in one inbred line characterised primarily by developmental arrest as pre-morphogenesis blastulae or gastrulae. The inadvertent selection for specific lethal mutations in the inbred line could be of interest, because if it is determined by a small number of major lethal genetic

mutations, the clarification of its molecular basis could lead to the development of a potential target mechanism for nematode control.

Multiple anthelmintic resistance will inevitably prove to be a major constraint to economic sheep production within the foreseeable future, and sensitive and specific markers are urgently required for the development of strategies that can be used to manage the problem. While current PCR based molecular methods are not sufficiently economic, robust or practical for application in veterinary practice, the development of new technologies such as automated, high-throughput screening may prove to be timely, enabling the field application of molecular markers for anthelmintic resistance. The currently available panel of unmapped *H. contortus* microsatellites is inadequate to identify linkage to genes or mutations conferring anthelmintic resistance in the progeny of the backcrossing experiments. The resources created by the genetic crossing methods that have been developed in this thesis may therefore be ahead of their time, but will become valuable assets once a fully sequenced annotated *H. contortus* genome is published and a larger panel of mapped neutral molecular markers becomes available, or whole nematode genome screening becomes practical and affordable. In the meantime, these resources will be nonetheless useful for current molecular biological study.

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Egg No.	Hcms 25		Hcms 27		Hcms 36		Hcms 120		Hcms 22co3		Hcms 8a20		18210		3561	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
1	213	219	358	358	152	152	297	297	250	250	196	240	218	218	285	285
2	209	209	358	358	152	152	285	285	250	250			218	218		
3	209	209			152	152			250	250	232	232	218	218	285	285
4					148	152	297	297	250	250	232	232				
6													218	218	285	285
7	209	219	358	358	148	148	297	297	250	250	192	240	218	218	285	285
8							285	285	250	250	232	232	218	218		
10	213	213	358	358	148	152	285	285	234	234	192	192	218	218	285	285
11	211	211	358	358	148	152	285	285	250	250	232	240	218	218	262	288
12	211	211														
13													218	218		
14	211	211			152	152			234	234	192	192	218	218	262	285
15	215	215	358	358	148	148	285	285	234	234	192	232	218	218	285	285
16	211	211			148	148			250	250	192	192				
17	211	211			148	148			250	250	196	196			285	285
18	213	213	358	358	152	152	285	285	250	250	240	240	218	218		
19	219	219			152	152	285	285	250	250					288	288
20	213	213													285	285
21					152	152	285	297			232	232				
22	213	213	358	358	148	148	297	297	250	250	232	232				
23	213	217	358	358	148	152	285	285	234	234	192	232	218	218		
24	211	213	358	358	148	148	297	297	250	250	196	232	218	218	262	288
25	209	209	358	358	148	152	285	285	250	250	232	232	218	218	288	288
26	219	219	358	358	152	152	297	297	250	250	240	240	218	218		
27	213	213			152	152	285	285			232	232	218	218	285	285
28	209	209					297	297	250	250	232	232	218	218		
29	215	215			148	148			234	234	232	232	218	218		
30	211	213	358	358	148	152	297	297	250	250	232	232	218	218	285	285
31					152	152					192	192				
32					148	148										
33	209	219	358	358	148	148	297	297	250	250			218	218	285	285
34							297	297	250	250						
35	213	213	358	358	152	152	285	285	250	250	192	192				
36	211	211	358	358	148	148			250	250					262	262
37	213	213	358	358	148	148	285	297	250	250					288	288
38																
39	211	211	358	358	148	148	297	297	250	250	232	232	218	218	285	285
40	213	219	358	358					250	250	240	240	218	218	285	285
41	211	211	358	358							232	232				
42	211	211	358	358	148	148	297	297	250	250	232	232	224	224	262	262
43	211	219	358	358	148	148	285	285	234	234			218	218	262	285
44	213	213	358	358	148	148	285	297			232	232	218	218	285	285
45					152	152	285	285								
46	217	217	358	358	148	152	285	285	234	234	192	192	218	218	285	285
47	211	211	358	358	148	152	285	285	250	250	232	240	218	218		
48	211	213	358	358	148	152	285	285	250	250	192	240	218	218	285	288
49	213	213	358	358	152	152	285	285	250	250					288	288
50	211	211	358	358	148	148			250	250	196	196	218	218		
51	213	213	358	358	148	148	285	285	250	250			218	218		
52											240	240				
53	213	213									232	232				
54	211	211	358	358	148	148	297	297	250	250	232	240	218	218	262	262
55	209	219	358	358	148	148	297	297	250	250	192	240	218	218	285	285
56																
57	209	209	358	358	148	152	285	285	250	250	232	232	218	218	262	288
58															262	262
59	219	219	358	358	148	148	297	297	250	250	184	240	218	218	285	285
60													218	218	285	285
61													218	218		
62	213	213	358	358	148	148	297	297	250	250	240	240	218	218	285	285
63	209	209	358	358	148	148			250	250	192	192	218	218		
64	211	211			148	148					192	192	218	218	262	262
65	213	213			152	152	285	297	234	234	232	232	218	218	262	262
66	209	209	358	358	152	152	285	285	250	250	232	232	218	218	285	285
67	217	217	358	358	148	148	297	297	250	250	232	232	224	224	262	262
68	219	219	358	358	152	152	285	285	250	250	192	240	218	218		
69	213	215	358	358	148	152	285	285	234	234	192	232	218	218	285	285
70											240	240				
71	219	219	358	358	148	148			250	250	192	192				
72	211	211	358	358	152	152	285	285	250	250	232	240	218	218	262	262
73	211	219	358	358	148	148	285	285					218	218	262	262
74	211	217	358	358	148	148	285	285	234	234			218	218	262	285
75					152	152							218	218	285	285
76	211	211	358	358	152	152			250	250	192	232			262	262
77	209	209	358	358			297	297			240	240	218	218	262	262
78																
79	213	213	358	358	148	148	285	285	250	250	232	232	218	218	285	285
80			358	358							232	232				
81	213	215	358	358	148	152	285	285	234	234	192	192	218	218	285	285
82	211	211	358	358	148	148			250	250	192	192			288	288
83	211	219	358	358	148	152	285	285	250	250	232	232	218	218	285	285
84	211	217	358	358	148	148	285	285	234	234			218	218	262	262
85	213	213	358	358	148	148	285	285	250	250					288	288
86	211	213	358	358	148	152	297	297			232	240				
87	211	211														
88	209	211							250	250	192	192	218	218	285	285
Heterozygotes	**17**		**0**		**14**		**4**		**0**		**16**		**0**		**7**	
Homozygotes	**50**		**53**		**50**		**49**		**59**		**43**		**55**		**44**	
PCR failure	**19**		**33**		**22**		**33**		**27**		**27**		**31**		**35**	

Appendix 3.1i): Autosomal microsatellite alleles for individual unfertilised eggs that had been shed by female *H. contortus* which had been surgically transferred as day 14 late L_4/immature adults into the abomasum of recipient Y. Heterozygous loci are highlighted.

Egg No.	X146945		X240993		X142509	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
1	151	151	239	239	179	179
2	151	151	239	239	180	180
3	151	151	245	245	179	179
4						
6	151	151	239	239	180	180
7	151	151	239	239	179	179
8	151	151			180	180
10	151	151	245	245	179	179
11	151	151	239	239	180	180
12						
13	151	151				
14	151	151	239	239	179	180
15	151	151	239	245	180	180
16	151	151	245	245	180	180
17	147	147	241	241	178	178
18	147	147	241	241	178	178
19	151	151	239	239	179	179
20						
21	151	151	239	245		
22						
23	151	151	245	245	179	180
24	147	151	239	241	178	179
25	151	151	239	239	179	179
26	151	151			180	180
27	147	151	245	245	179	179
28	151	151	241	241	179	179
29	151	151				
30	151	151	239	239	179	180
31	151	151			179	179
32						
33	151	151	239	239	179	179
34					179	179
35						
36	147	147	241	241	178	178
37	151	151	239	239	179	179
38	147	151				
39	151	151	239	239	180	180
40	151	151	239	239	179	179
41	151	151				
42	151	151	239	239	179	179
43	151	151	239	239	179	179
44	151	151	239	239	180	180
45	151	151	239	239	179	179
46	151	151	245	245	179	180
47	151	151	239	239	180	180
48	147	147	239	239	178	180
49	151	151	239	239	179	179
50	147	151	239	241	178	178
51	151	151	239	239	180	180
52	151	151	239	239	178	178
53	151	151				
54	151	151	239	239	180	180
55	151	151	239	239	179	179
56						
57	147	151	239	239	178	178
58						
59	151	151	239	239	179	179
60	147	151	239	241	178	180
61			239	239		
62	151	151	239	239	179	179
63	151	151	239	239	179	179
64	151	151	239	239	180	180
65						
66	151	151	239	239	179	179
67	151	151	239	239	180	180
68	151	151	239	239	180	180
69	151	151	239	245	180	180
70	151	151				
71	147	147				
72	151	151	239	245	179	179
73	151	151	239	239	179	179
74	151	151	239	239	179	179
75	151	151	245	245	179	179
76					179	179
77	151	151	245	245	179	179
78						
79	151	151	239	239	180	180
80	147	151	239	239		
81	151	151	239	245	180	180
82	151	151	239	245	179	179
83	151	151	245	245	179	179
84	151	151	239	239	179	179
85	151	151	239	239	179	179
86	151	151				
87						
88			239	239		
Heterozygotes	**7**		**9**		**7**	
Homozygotes	**64**		**53**		**56**	
PCR failure	**15**		**24**		**23**	

Appendix 3.1ii): X chromosome microsatellite alleles for individual unfertilised eggs that had been shed by female *H. contortus* which had been surgically transferred as day 14 late L_4/immature adults into the abomasum of recipient Y. Heterozygous loci are highlighted.

ID	Line	8a20		Hcms33		Hcms25		Hcms27		Hcms40		22co3	
3289A	ISExWRS	208	232	204	204	211	213	358	358	295	297	250	250
3289B	ISExWRS	240	248	218	218	213	217	358	358	0	0	242	242
3289C	ISExWRS	196	240	218	218	207	210	358	358	285	285	234	250
3289D	ISExWRS	240	240	0	0	213	217	358	358	285	285	234	234
3289E	ISExWRS	232	232	204	204	209	213	358	358	285	297	250	250
3289F	ISExWRS	196	232	218	218	211	215	358	358	285	285	250	250
3289G	ISExWRS	232	232	204	218	209	209	358	358	297	297	250	250
3289H	ISExWRS	240	240	204	218	213	213	358	358	285	297	250	250
3289I	ISExWRS	196	240	218	218	211	215	358	358	285	297	250	250
3289J	ISExWRS	192	240	204	204	213	213	358	358	0	0	0	0
3289K	ISExWRS	232	240	204	218	211	213	358	358	297	297	250	250
3289L	ISExWRS	0	0	204	218	211	217	358	358	285	285	250	250
3289M	ISExWRS	244	248	204	204	207	207	344	344	295	295	242	242
3289O	ISExWRS	232	248	204	204	207	209	338	338	295	297	250	250
3289P	ISExWRS	196	196	218	218	209	217	358	358	297	297	250	250
3289Q	ISExWRS	240	248	204	204	0	0	358	358	295	297	242	242
3289R	ISExWRS	232	248	204	204	211	215	358	358	297	297	250	250
3289S	ISExWRS	196	240	204	204	213	213	358	358	0	0	234	250
3289T	ISExWRS	232	232	204	218	211	213	358	358	295	297	250	250
3289U	ISExWRS	240	240	218	218	213	213	338	358	297	297	242	250
3289V	ISExWRS	240	240	204	218	205	213	338	338	0	0	258	258
3289W	ISExWRS	232	240	204	218	213	213	358	358	297	297	250	250
3289X	ISExWRS	240	240	218	218	211	213	338	338	297	297	250	250
3289Y	ISExWRS	240	248	204	218	211	213	0	0	0	0	234	234
3289Z	ISExWRS	240	240	204	218	213	217	338	338	285	285	250	250
3289AA	ISExWRS	196	208	204	204	213	213	358	358	295	295	250	250
3289AB	ISExWRS	196	232	204	218	213	213	358	358	285	285	250	250
3289AC	ISExWRS	192	192	204	218	209	211	358	358	0	0	250	250
3289AD	ISExWRS	196	196	218	218	211	213	358	358	297	297	250	250
GL2097	ISE	232	232	218	218	215	215	0	0	0	0	250	250
GL2100	ISE	240	240	204	204	211	213	358	358	297	297	250	250
GL2102	ISE	232	240	0	0	211	213	358	358	0	0	250	250
GL2103	ISE	240	240	204	204	211	213	358	358	297	297	250	250
GL2104	ISE	240	240	218	218	211	213	0	0	285	285	0	0
GL2105	ISE	280	283	204	204	0	0	0	0	285	285	250	250
GL2106	ISE	232	240	204	218	211	211	358	358	297	297	250	250
GL2107	ISE	196	232	204	204	217	217	358	358	297	297	250	250
GL2109	ISE	232	232	204	218	0	0	0	0	297	297	250	250
GL2110	ISE	240	240	218	218	213	213	358	358	285	297	250	250
GL2111	ISE	240	240	204	218	0	0	358	358	297	297	0	0
GL2113	ISE	232	240	218	218	213	213	358	358	285	297	250	250
GL2114	ISE	232	232	204	204	209	217	358	358	297	297	250	250
GL2115	ISE	232	232	218	218	211	211	358	358	297	297	250	250
GL2116	ISE	196	196	204	218	0	0	358	358	297	297	0	0
GL2117	ISE	0	0	204	218	213	213	358	358	285	285	250	250
GL2118	ISE	192	232	204	204	211	217	358	358	285	285	250	250
GL2119	ISE	0	0	218	218	211	211	358	358	285	297	0	0
GL2120	ISE	232	232	204	218	211	211	358	358	285	285	250	250
GL2122	ISE	240	240	218	218	211	213	358	358	285	297	0	0
GL2123	ISE	232	232	204	218	213	213	358	358	297	297	250	250
GL2126	ISE	232	232	204	218	213	213	358	358	285	297	250	250
GL2153	WRS	196	196	204	204	211	211	358	358	0	0	0	0
GL2154	WRS	240	240	204	204	207	207	0	0	0	0	234	234
GL2155	WRS	240	248	0	0	213	213	338	338	297	297	242	242
GL2156	WRS	240	248	204	204	215	215	0	0	295	295	0	0
GL2161	WRS	192	232	218	218	217	217	0	0	285	285	250	250
GL2164	WRS	240	248	218	218	207	211	0	0	279	279	242	242
GL2165	WRS	240	240	204	204	0	0	358	358	295	295	0	0
GL2170	WRS	179	248	204	204	0	0	338	338	0	0	242	242
GL2172	WRS	240	240	204	204	213	213	344	358	0	0	0	0
GL2176	WRS	240	240	218	218	213	213	0	0	285	285	250	250
GL2181	WRS	240	248	204	204	213	213	358	358	297	297	0	0

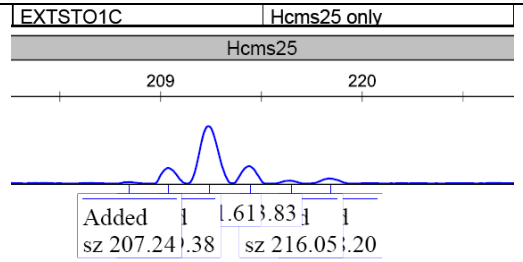
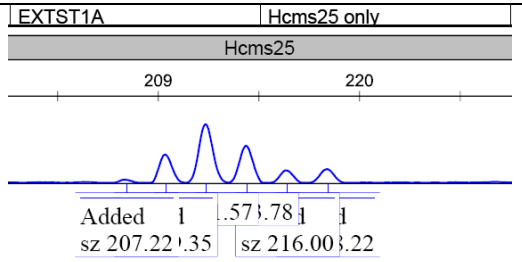
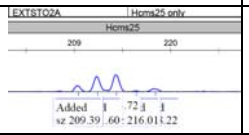
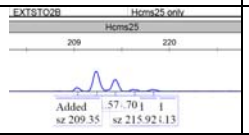
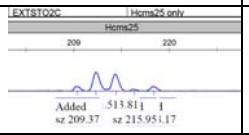
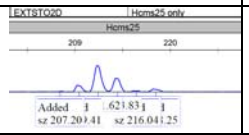
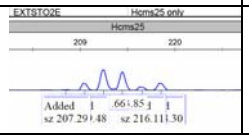
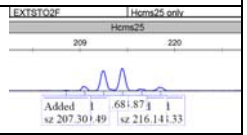
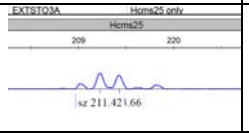
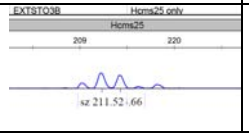
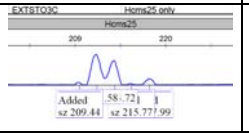
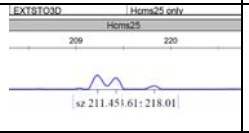
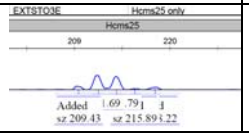
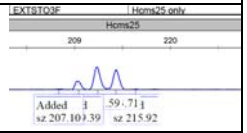
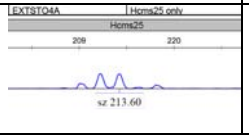
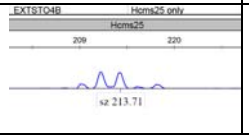
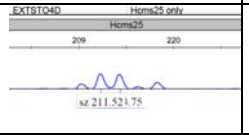
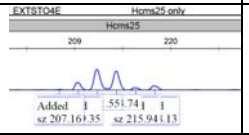
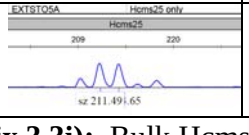
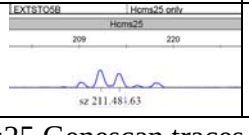
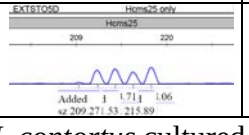
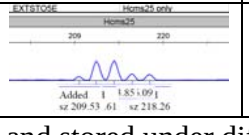
Appendix 3.2i): Microsatellite alleles of individual eggs (ISE x WRS) harvested 28 days after donor infection with L_3 and individual L_3 (ISE and WRS) used to generate PCA plots to investigate interbreeding between populations of MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus*.

ID	Line	8a20		Hcms33		Hcms25		Hcms27		Hcms40		22co3	
3134A	ISExCAVR	232	248	204	204	213	213	338	358	285	297	258	258
3134B	ISExCAVR	0	0	204	204	211	213	358	358	285	285	250	258
3134C	ISExCAVR	214	240	204	218	215	215	358	358	285	297	234	250
3134D	ISExCAVR	240	240	204	204	211	211	0	0	0	0	250	250
3134E	ISExCAVR	232	240	218	218	211	217	358	358	297	297	250	250
3134F	ISExCAVR	232	232	218	218	211	213	358	358	285	285	250	250
3134G	ISExCAVR	196	232	218	218	211	211	358	358	297	297	250	250
3134H	ISExCAVR	240	240	204	218	213	213	358	358	285	285	250	250
3134I	ISExCAVR	0	0	204	204	211	213	344	344	285	297	250	250
3134J	ISExCAVR	0	0	204	204	213	213	338	358	285	285	250	250
3134K	ISExCAVR	240	248	204	218	211	217	358	358	297	297	250	258
3134L	ISExCAVR	192	192	218	218	213	213	358	358	285	285	0	0
3134M	ISExCAVR	232	232	204	204	211	213	358	358	297	297	250	250
3134N	ISExCAVR	240	240	204	218	213	213	358	358	285	285	0	0
3134O	ISExCAVR	196	196	204	204	213	213	358	358	285	285	250	250
3134P	ISExCAVR	232	232	204	218	211	213	358	358	285	285	250	250
3134Q	ISExCAVR	192	208	204	204	211	213	338	358	285	297	250	250
3134R	ISExCAVR	196	196	204	218	211	217	358	358	297	297	250	250
3134U	ISExCAVR	240	248	204	218	213	213	358	358	297	297	250	250
3134V	ISExCAVR	196	196	204	218	211	213	358	358	285	285	250	250
3134W	ISExCAVR	248	248	204	218	211	211	358	358	297	297	250	250
3134X	ISExCAVR	232	248	204	204	209	213	358	358	297	297	234	234
3134AA	ISExCAVR	232	240	204	204	211	211	358	358	285	297	250	250
3134AB	ISExCAVR	212	240	204	218	0	0	358	358	297	297	250	250
3134AC	ISExCAVR	0	0	204	204	213	213	338	358	0	0	250	250
3134AD	ISExCAVR	196	196	204	218	211	211	358	358	285	285	250	250
GL2097	ISE	232	232	218	218	215	215	0	0	0	0	250	250
GL2100	ISE	240	240	204	204	211	213	358	358	297	297	250	250
GL2102	ISE	232	240	0	0	211	213	358	358	0	0	250	250
GL2103	ISE	240	240	204	204	211	213	358	358	297	297	250	250
GL2104	ISE	240	240	218	218	211	213	0	0	285	285	0	0
GL2105	ISE	280	283	204	204	0	0	0	0	285	285	250	250
GL2106	ISE	232	240	204	218	211	211	358	358	297	297	250	250
GL2107	ISE	196	232	204	204	217	217	358	358	297	297	250	250
GL2109	ISE	232	232	204	218	0	0	0	0	297	297	250	250
GL2110	ISE	240	240	218	218	213	213	358	358	285	297	250	250
GL2111	ISE	240	240	204	218	0	0	358	358	297	297	0	0
GL2113	ISE	232	240	218	218	213	213	358	358	285	297	250	250
GL2114	ISE	232	232	204	204	209	217	358	358	297	297	250	250
GL2115	ISE	232	232	218	218	211	211	358	358	297	297	250	250
GL2116	ISE	196	196	204	218	0	0	358	358	297	297	0	0
GL2117	ISE	0	0	204	218	213	213	358	358	285	285	250	250
GL2118	ISE	192	232	204	204	211	217	358	358	285	285	250	250
GL2119	ISE	0	0	218	218	211	211	358	358	285	297	0	0
GL2120	ISE	232	232	204	218	211	211	358	358	285	285	250	250
GL2122	ISE	240	240	218	218	211	213	358	358	285	297	0	0
GL2123	ISE	232	232	204	218	213	213	358	358	297	297	250	250
GL2126	ISE	232	232	204	218	213	213	358	358	285	297	250	250
GL2236	CAVR	240	248	204	204	213	213	0	0	0	0	250	250
GL2237	CAVR	248	248	204	204	0	0	338	338	297	297	0	0
GL2239	CAVR	196	248	204	218	213	213	338	338	0	0	250	250
GL2240	CAVR	240	240	204	204	213	213	0	0	0	0	250	2550
GL2241	CAVR	248	248	204	204	213	213	344	344	0	0	250	250
GL2246	CAVR	248	248	204	204	213	213	0	0	0	0	250	250
GL2247	CAVR	0	0	204	204	213	213	338	338	0	0	0	0
GL2248	CAVR	248	265	204	204	213	213	344	344	248	248	258	258
GL2249	CAVR	248	248	204	204	213	213	338	338	0	0	0	0

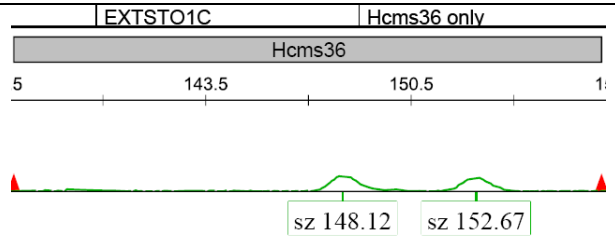
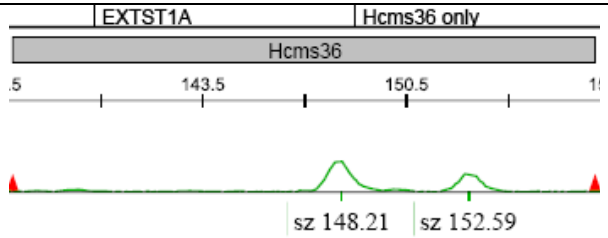
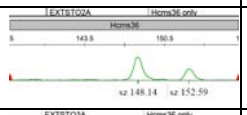
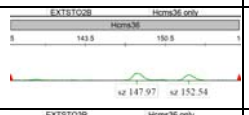
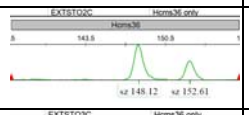
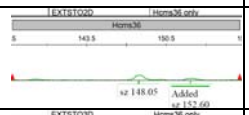
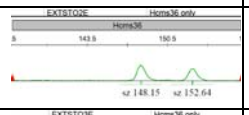
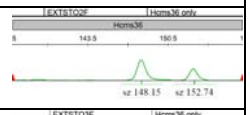
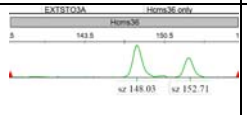
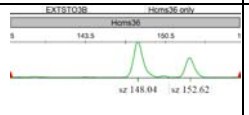
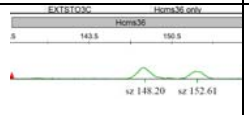
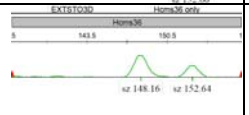
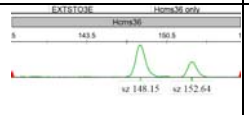
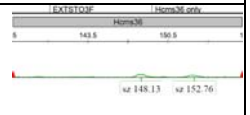
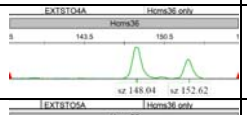
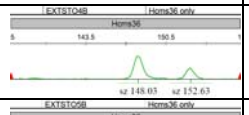
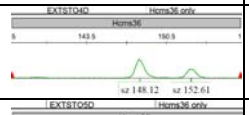
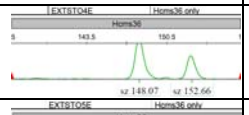
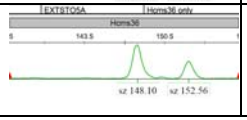
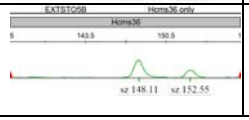
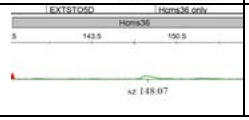
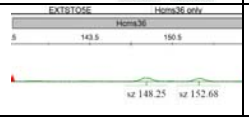
Appendix 3.2ii): Microsatellite alleles of individual eggs (ISE x CAVR) harvested 28 days after donor infection with L_3 and individual L_3 (ISE and CAVR) used to generate PCA plots to investigate interbreeding between populations of MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus*.

ID	Line	8a20		Hcms33		Hcms25		Hcms27		Hcms40		22co3	
3182A	WRSxCAVR	196	248	204	218	215	217	338	338	297	297	250	250
3182B	WRSxCAVR	196	196	204	218	213	213	338	358	0	0	250	250
3182C	WRSxCAVR	196	248	204	204	213	213	358	358	0	0	250	250
3182D	WRSxCAVR	0	0	204	218	213	213	338	338	295	297	0	0
3182F	WRSxCAVR	196	196	204	204	211	211	338	338	0	0	250	250
3182G	WRSxCAVR	196	196	204	218	215	217	338	358	0	0	250	250
3182H	WRSxCAVR	0	0	204	218	207	211	344	344	297	297	0	0
3182I	WRSxCAVR	240	248	204	218	213	213	344	344	0	0	242	242
3182J	WRSxCAVR	212	256	204	204	209	213	344	344	0	0	250	250
3182K	WRSxCAVR	196	240	218	218	205	205	358	358	0	0	0	0
3182L	WRSxCAVR	240	248	204	204	213	213	344	344	0	0	250	250
3182M	WRSxCAVR	232	232	0	0	213	213	338	358	0	0	250	250
3182O	WRSxCAVR	240	248	204	218	207	213	358	358	0	0	0	0
3182P	WRSxCAVR	220	240	204	218	213	213	0	0	295	297	250	250
3182Q	WRSxCAVR	240	256	204	204	205	213	338	338	295	297	0	0
3182R	WRSxCAVR	240	240	204	204	213	217	358	358	0	0	0	0
3182S	WRSxCAVR	248	248	204	204	213	213	358	358	297	297	0	0
3182T	WRSxCAVR	196	240	204	204	211	217	0	0	297	297	242	242
3182U	WRSxCAVR	248	248	204	204	213	213	0	0	295	295	250	250
3182V	WRSxCAVR	240	240	204	204	215	217	338	338	0	0	250	250
3182W	WRSxCAVR	212	248	204	204	211	213	338	338	0	0	234	250
3182Y	WRSxCAVR	240	248	204	204	213	215	338	338	295	295	0	0
3182AA	WRSxCAVR	196	240	204	204	211	217	338	344	0	0	250	250
3182AB	WRSxCAVR	0	0	0	0	211	213	358	358	285	285	250	250
3182AC	WRSxCAVR	240	248	218	218	213	213	338	338	0	0	250	250
3182AD	WRSxCAVR	240	248	204	218	207	213	0	0	0	0	250	250
GL2153	WRS	196	196	204	204	211	211	358	358	0	0	0	0
GL2154	WRS	240	240	204	204	207	207	0	0	0	0	234	234
GL2155	WRS	240	248	0	0	213	213	338	338	297	297	242	242
GL2156	WRS	240	248	204	204	215	215	0	0	295	295	0	0
GL2161	WRS	192	232	218	218	217	217	0	0	285	285	250	250
GL2164	WRS	240	248	218	218	207	211	0	0	279	279	242	242
GL2165	WRS	240	240	204	204	0	0	358	358	295	295	0	0
GL2170	WRS	179	248	204	204	0	0	338	338	0	0	242	242
GL2172	WRS	240	240	204	204	213	213	344	358	0	0	0	0
GL2176	WRS	240	240	218	218	213	213	0	0	285	285	250	250
GL2181	WRS	240	248	204	204	213	213	358	358	297	297	0	0
GL2236	CAVR	240	248	204	204	213	213	0	0	0	0	250	250
GL2237	CAVR	248	248	204	204	0	0	338	338	297	297	0	0
GL2239	CAVR	196	248	204	218	213	213	338	338	0	0	250	250
GL2240	CAVR	240	240	204	204	213	213	0	0	0	0	250	2550
GL2241	CAVR	248	248	204	204	213	213	344	344	0	0	250	250
GL2246	CAVR	248	248	204	204	213	213	0	0	0	0	250	250
GL2247	CAVR	0	0	204	204	213	213	338	338	0	0	0	0
GL2248	CAVR	248	265	204	204	213	213	344	344	248	248	258	258
GL2249	CAVR	248	248	204	204	213	213	338	338	0	0	0	0

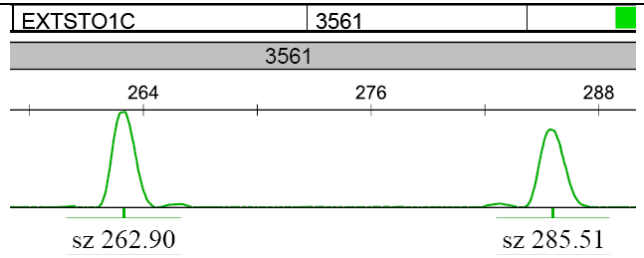
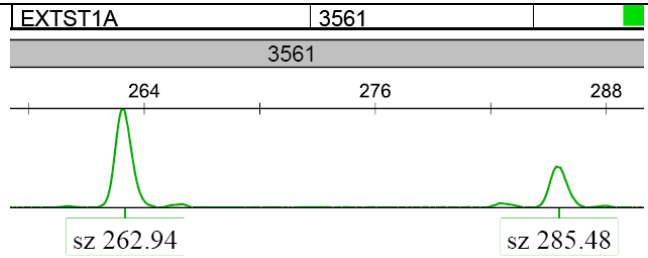
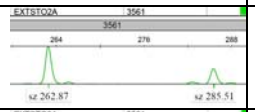
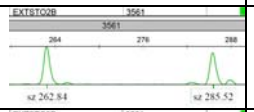
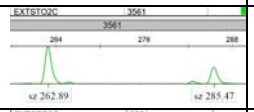
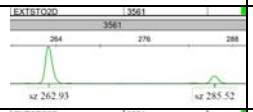
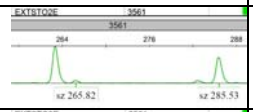
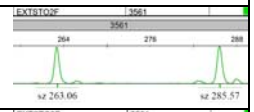
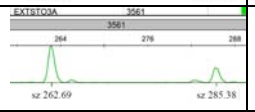
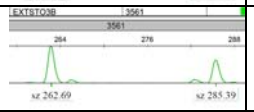
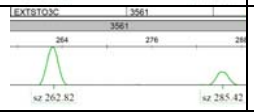
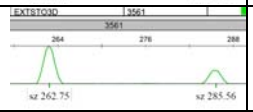
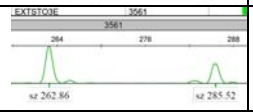
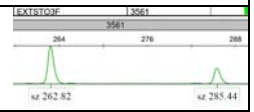
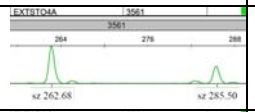
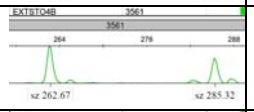
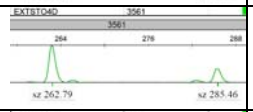
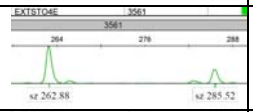
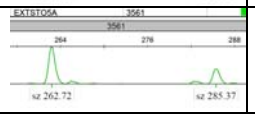
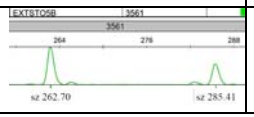
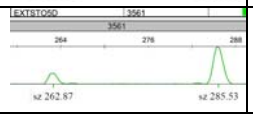
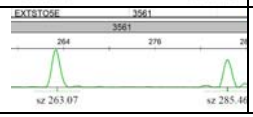
Appendix 3.2iii): Microsatellite alleles of individual eggs (WRS x CAVR) harvested 28 days after donor infection with L_3 and individual L_3 (WRS and CAVR) used to generate PCA plots to investigate interbreeding between populations of MHco4 (WRS) and MHco10 (CAVR) strains of *H. contortus*.

Hcms25	20°C incubation			37°C incubation		
Day 10						
	4°C store	22°C store	37°C store	4°C store	22°C store	37°C store
Day 24						
Day 58						
Day 116			L_3 all dead			L_3 all dead
Day 212			L_3 all dead			L_3 all dead

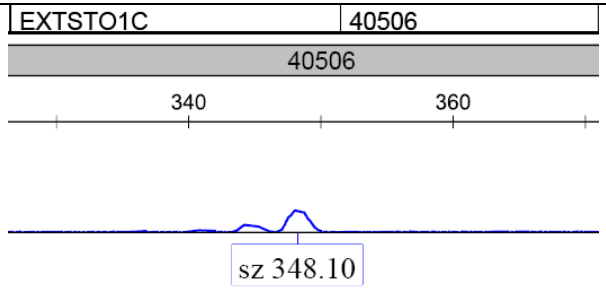
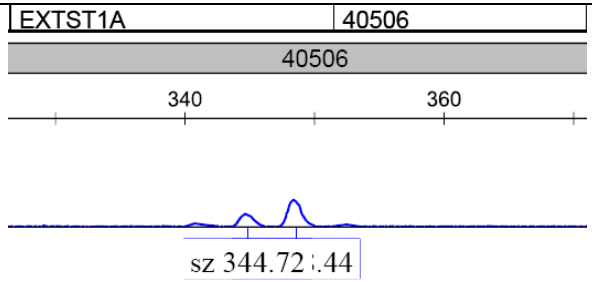
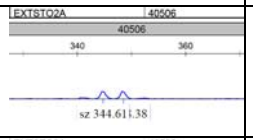
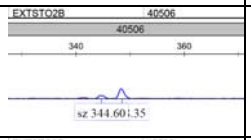
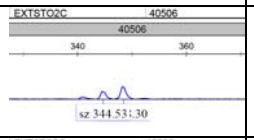
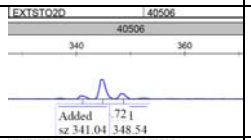
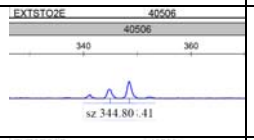
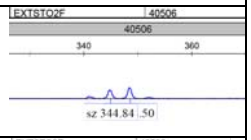
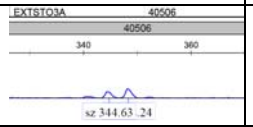
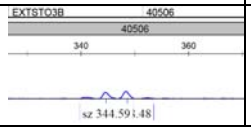
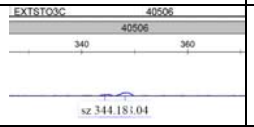
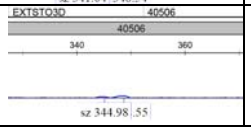
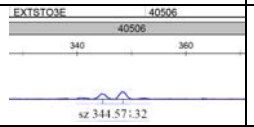
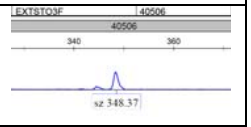
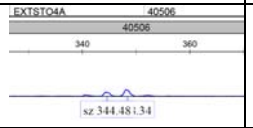
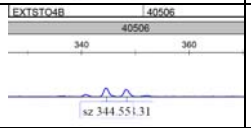
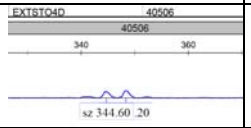
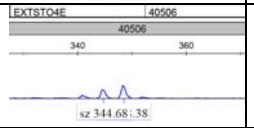
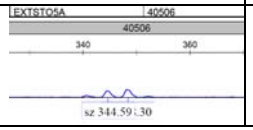
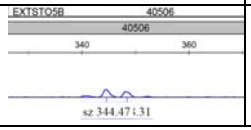
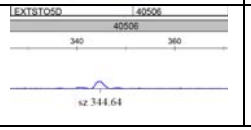
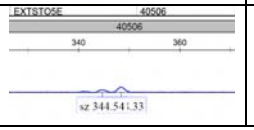
Appendix 3.3i): Bulk Hcms25 Genescan traces for MHco3 (ISE) *H. contortus* cultured and stored under different conditions

Hcms36	**20°C incubation**			**37°C incubation**		
Day 10						
	4°C store	22°C store	37°C store	4°C store	22°C store	37°C store
Day 24						
Day 58						
Day 116			L_3 all dead			L_3 all dead
Day 212			L_3 all dead			L_3 all dead

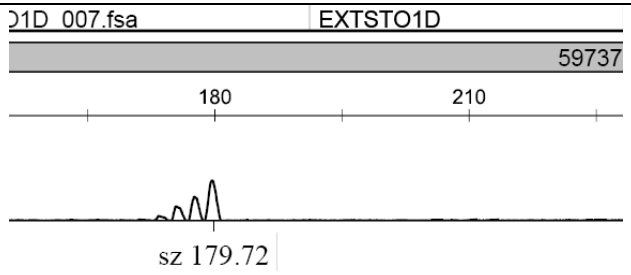
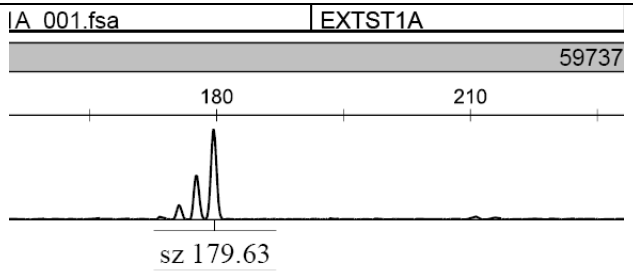
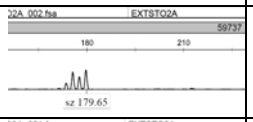
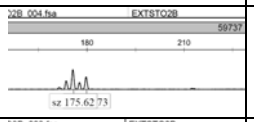
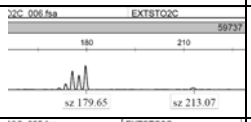
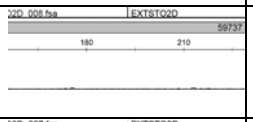
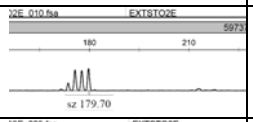
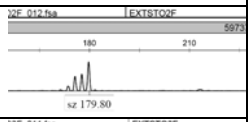
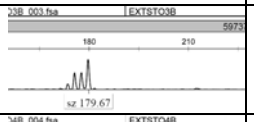
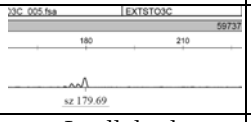
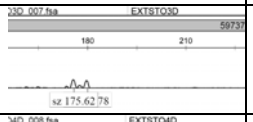
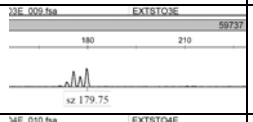
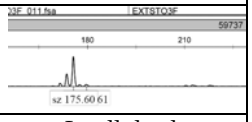
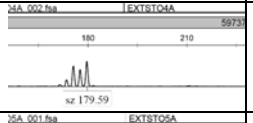
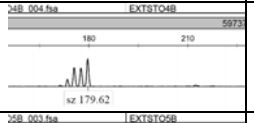
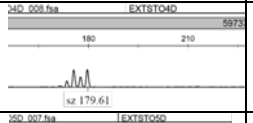
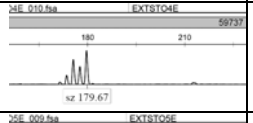
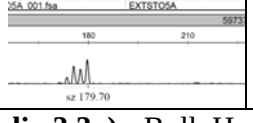
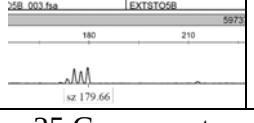
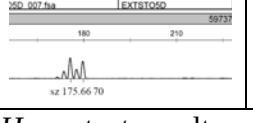
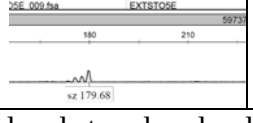
Appendix 3.3ii): Bulk Hcms36 Genescan traces for MHco3 (ISE) *H. contortus* cultured and stored under different conditions

3561	20°C incubation			37°C incubation		
Day 10						
	4°C store	22°C store	37°C store	4°C store	22°C store	37°C store
Day 24						
Day 58						
Day 116			L_3 all dead			L_3 all dead
Day 212			L_3 all dead			L_3 all dead

Appendix 3.3iii): Bulk 3561 Genescan traces for MHco3 (ISE) *H. contortus* cultured and stored under different conditions

40506	20°C incubation			37°C incubation		
Day 10						
	4°C store	22°C store	37°C store	4°C store	22°C store	37°C store
Day 24						
Day 58						
Day 116			L_3 all dead			L_3 all dead
Day 212			L_3 all dead			L_3 all dead

Appendix 3.3iv): Bulk 40506 Genescan traces for MHco3 (ISE) *H. contortus* cultured and stored under different conditions

59737	20°C incubation			37°C incubation		
Day 10						
	4°C store	22°C store	37°C store	4°C store	22°C store	37°C store
Day 24						
Day 58						
Day 116			L_3 all dead			L_3 all dead
Day 212			L_3 all dead			L_3 all dead

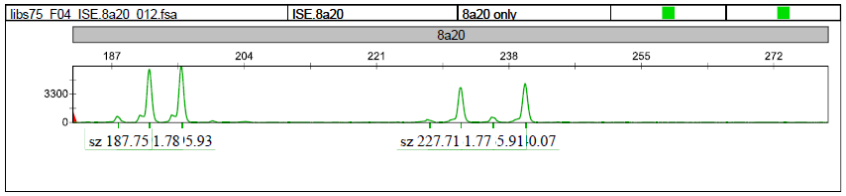
Appendix 3.3v): Bulk Hcms25 Genescan traces for MHco3 (ISE) *H. contortus* cultured and stored under different conditions

ID	Line	Hcms15		Hcms27		Hcms94		Hcms22co3	
8A	20 X 20	0	0	0	0	0	0	0	0
8B	20 X 20	272	287	0	0	233	233	250	250
8C	20 X 20	272	287	358	358	233	235	0	0
8D	20 X 20	272	287	358	358	233	235	258	258
8E	20 X 20	0	0	0	0	0	0	0	0
8F	20 X 20	272	287	344	358	233	233	234	250
8G	20 X 20	272	287	358	358	233	233	0	0
8H	20 X 20	272	287	358	358	233	233	0	0
9A	20 X 20	287	287	0	0	233	233	0	0
9B	20 X 20	287	287	0	0	233	233	250	250
9C	20 X 20	0	0	358	358	0	0	250	258
9D	20 X 20	287	287	0	0	0	0	0	0
9E	20 X 20	0	0	358	358	0	0	0	0
9F	20 X 20	272	287	358	358	233	233	250	258
9G	20 X 20	272	287	358	358	233	233	250	258
9H	20 X 20	287	287	0	0	233	233	0	0
10A	20 X 20	272	287	0	0	233	233	250	250
10B	20 X 20	272	287	358	358	233	233	250	250
10C	20 X 20	272	287	338	358	233	233	250	250
10D	20 X 20	0	0	0	0	0	0	250	250
10E	20 X 20	272	287	0	0	0	0	0	0
10F	20 X 20	272	287	358	358	233	233	0	0
10G	20 X 20	272	287	358	358	233	233	0	0
10H	20 X 20	272	287	344	358	233	233	250	250
11A	20 X 20	272	287	0	0	233	233	250	250
11B	20 X 20	0	0	344	358	0	0	234	234
11C	20 X 20	0	0	0	0	0	0	0	0
11D	20 X 20	0	0	0	0	0	0	0	0
11E	20 X 20	272	287	358	358	0	0	250	250
11F	20 X 20	272	287	344	344	233	233	234	234
11G	20 X 20	272	287	344	358	0	0	0	0
11H	20 X 20	272	287	344	358	233	233	242	250
12A	20 X 20	287	287	358	358	233	233	250	250
12B	20 X 20	287	287	344	344	233	233	0	0
12C	20 X 20	272	287	338	258	233	233	250	250
12D	20 X 20	272	287	358	358	233	233	0	0
12E	20 X 20	272	287	358	358	233	233	0	0
12F	20 X 20	272	287	0	0	233	233	0	0
12G	20 X 20	272	287	358	358	233	233	234	250
12H	20 X 20	272	287	358	358	0	0	0	0

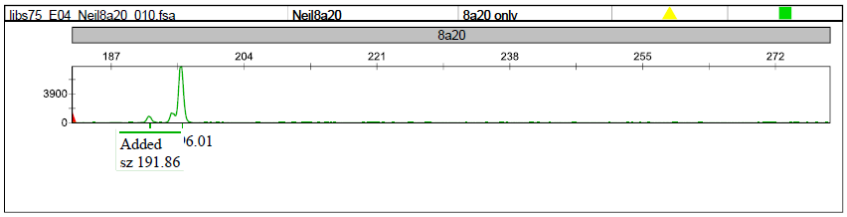
Appendix 3.4: Microsatellite genotypes of L_3 F_1 progeny of a genetic cross between 20 male MHco3 (ISE) and 20 female MHco4 (WRS) *H. contortus*.

Egg ID	3561		182762		240993		Hcms25		Hcms36	
E1	285	285	372	372	239	239	211	211	152	152
E2	261	261	372	372	239	239	217	217	152	152
E3	261	261	374	374	241	241	217	217	152	152
E4	261	261	372	372	239	239	217	217	nil	nil
E5	285	285	374	374	241	241	217	217	152	152
E6	285	285	372	374	239	241	217	217	152	152
E7	261	261	372	372	239	239	nil	nil	152	152
E8	261	261	374	374	241	241	217	217	nil	nil
E9	261	261	372	372	239	239	211	217	nil	nil
E10	261	261	374	374	241	241	217	217	152	152
E11	285	285	372	372	239	239	217	217	nil	nil
E12	261	261	372	374	239	241	217	217	148	148
E13	261	261	372	372	239	239	217	217	152	152
E14	285	285	374	374	241	241	211	211	nil	nil
E15	261	261	372	374	241	241	217	217	152	152
E16	261	285	372	372	239	241	217	217	148	148
E17	261	261	374	374	241	241	217	217	148	148
E18	285	285	372	372	239	239	217	217	nil	nil
E19	285	285	372	372	239	239	217	217	nil	nil
E20	285	285	372	372	239	239	217	217	152	152
LysC	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil

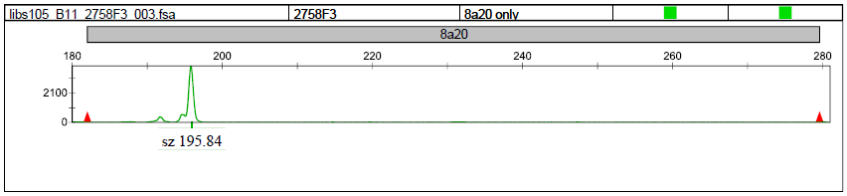
Appendix 4.1: Microsatellite genotypes of inbred MHco3.N1 F_4(2) eggs that did not hatch after 48 hours incubation.



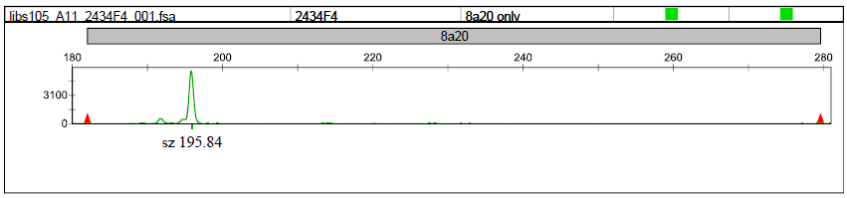
MHco3 (ISE)



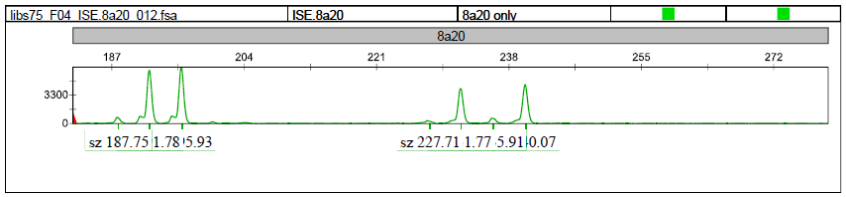
MHco3.N1 F_2



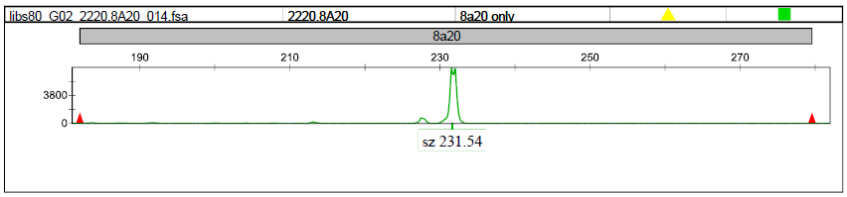
MHco3.N1 F_3



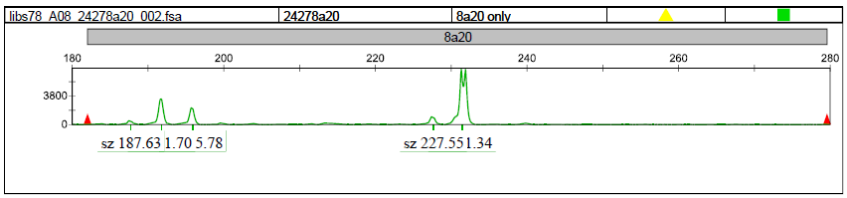
MHco3.N1 F_4



MHco3 (ISE)

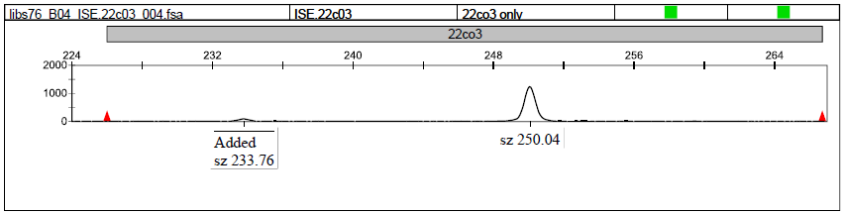


MHco3.N2 F_2

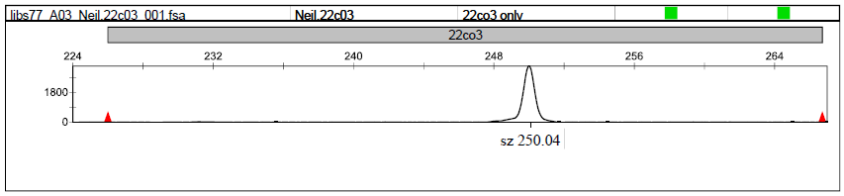


MHco3.N1 F_3

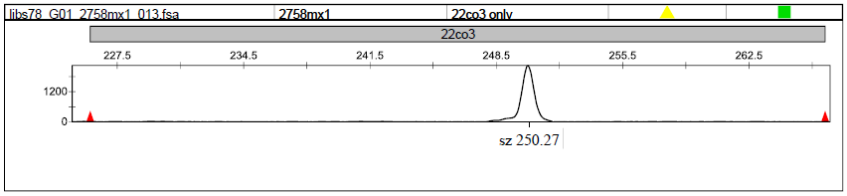
Appendix 4.2: Microsatellite Genescan traces for microsatellite marker Hcms8a20 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



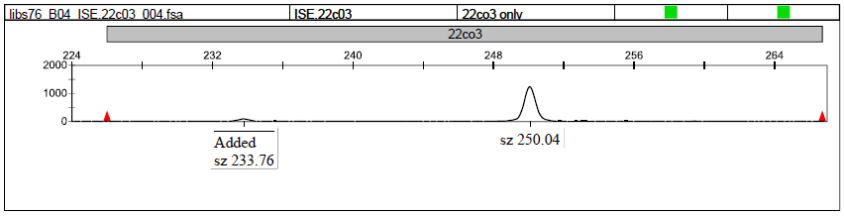
MHco3 (ISE)



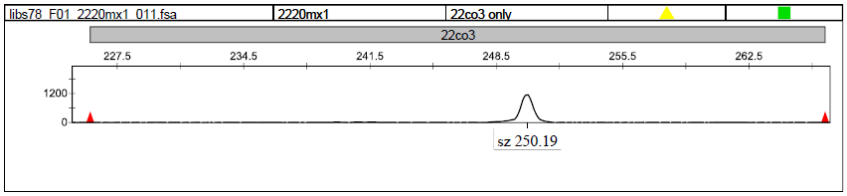
MHco3.N1 F_2



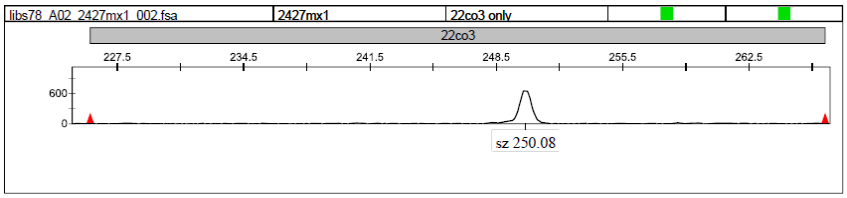
MHco3.N1 F_3



MHco3 (ISE)

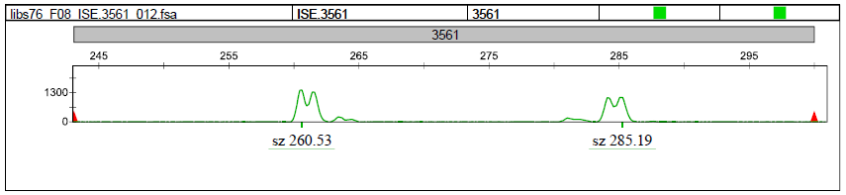


MHco3.N2 F_2

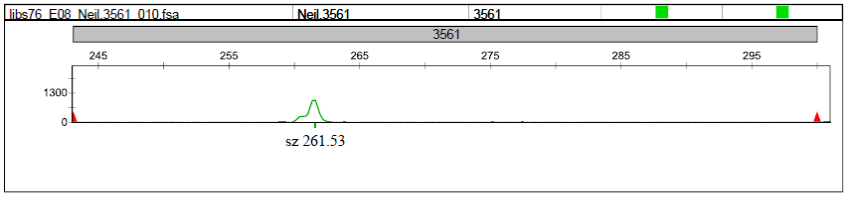


MHco3.N2 F_3

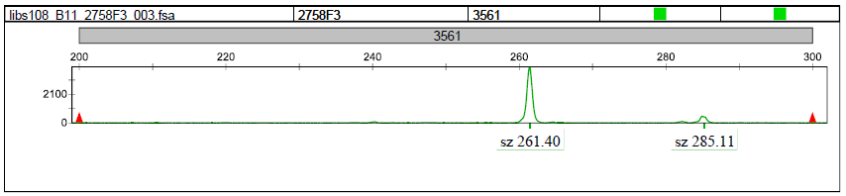
Appendix 4.2: Microsatellite Genescan traces for microsatellite marker Hcms22co3 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



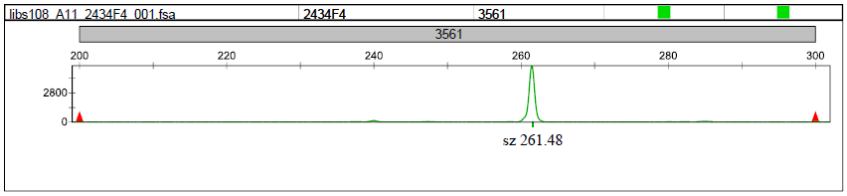
MHco3 (ISE)



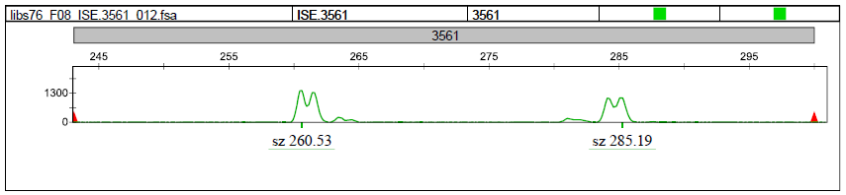
MHco3.N1 F_2



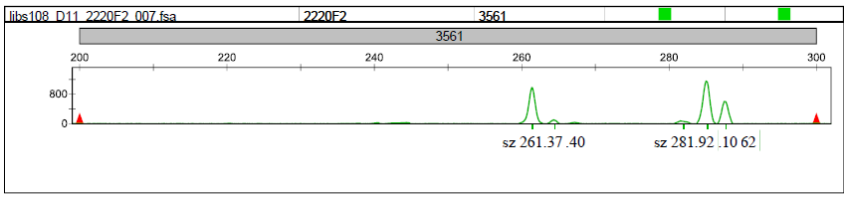
MHco3.N1 F_3



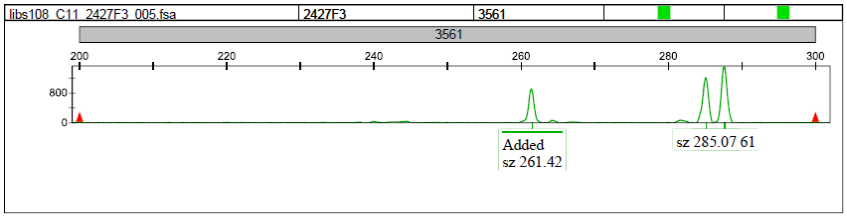
MHco3.N1 F_4



MHco3 (ISE)

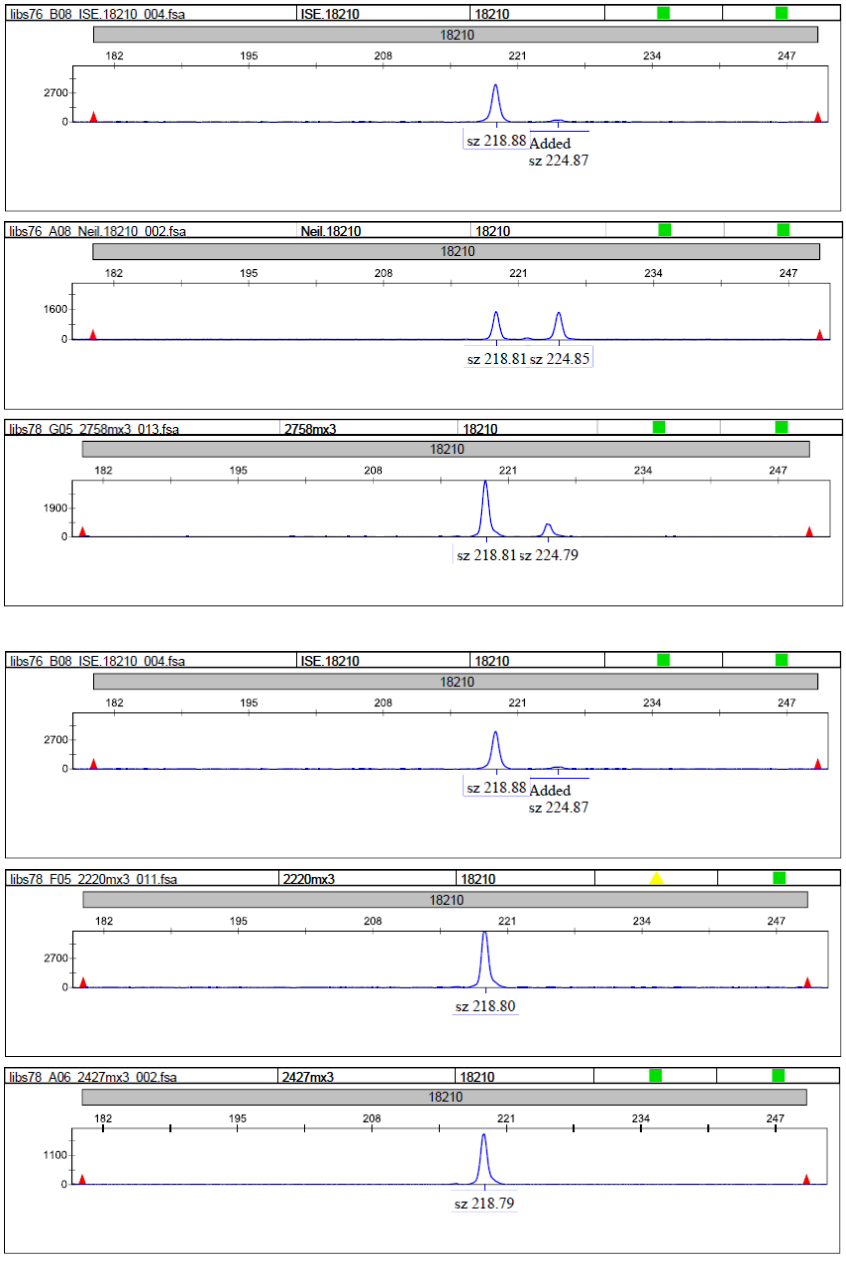


MHco3.N2 F_2



MHco3.N2 F_3

Appendix 4.2: Microsatellite Genescan traces for microsatellite marker 3561 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



MHco3 (ISE)

MHco3.N1 F_2

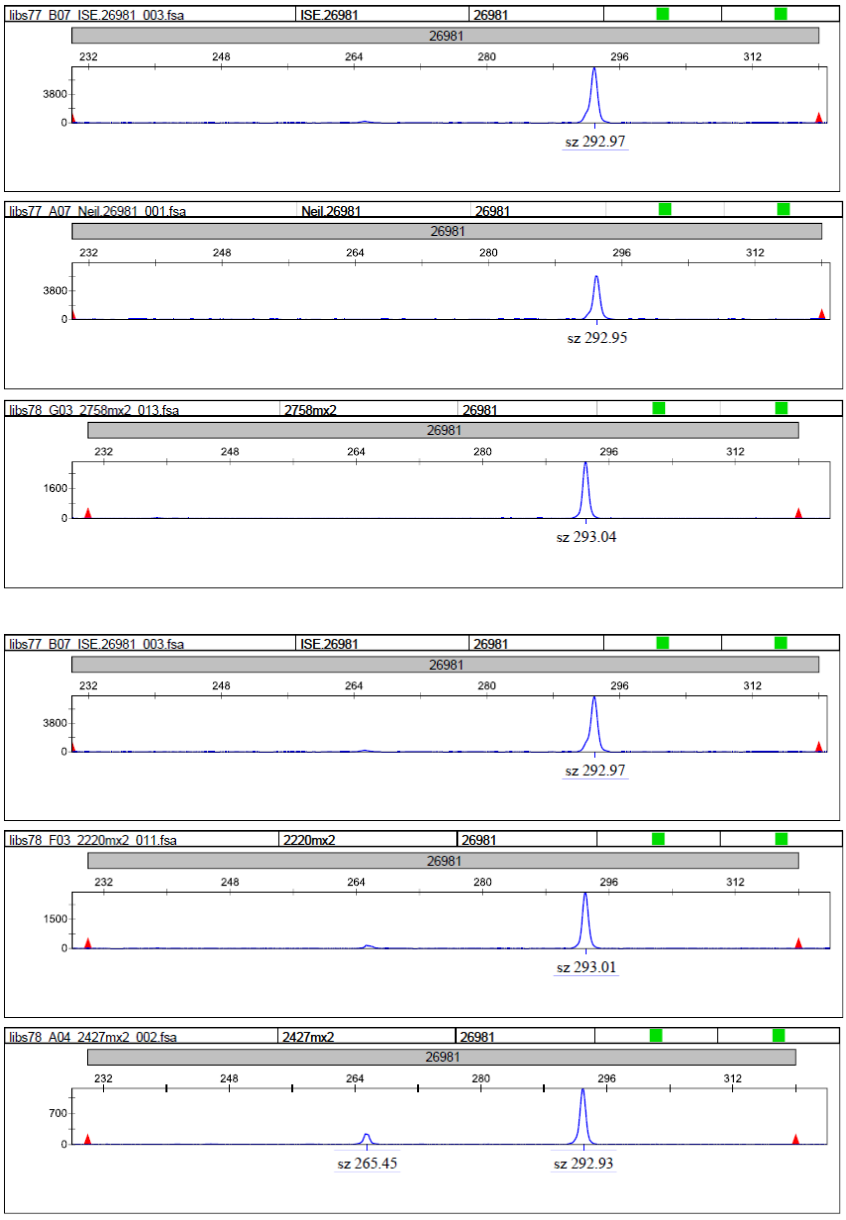
MHco3.N1 F_3

MHco3 (ISE)

MHco3.N2 F_2

MHco3.N2 F_3

Appendix 4.2: Microsatellite Genescan traces for microsatellite marker 18210 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



MHco3 (ISE)

MHco3.N1 F_2

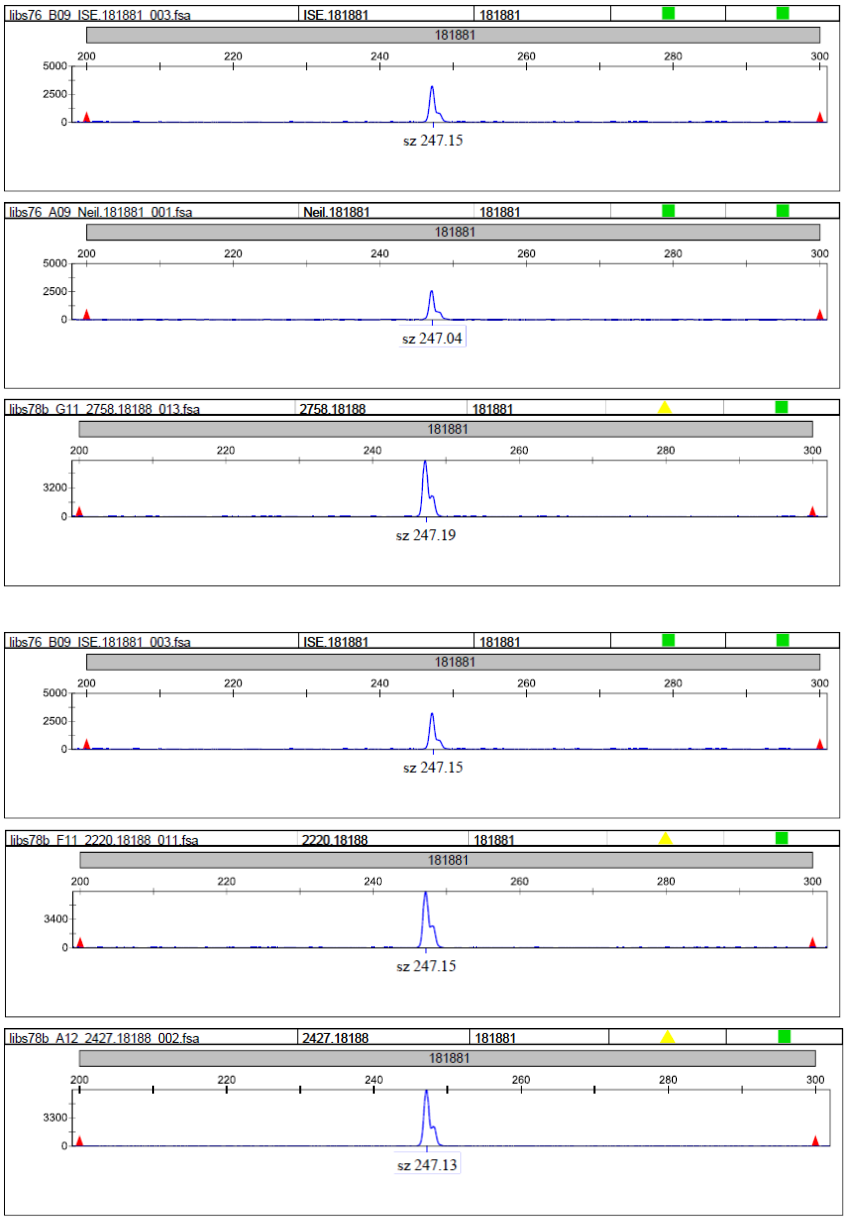
MHco3.N1 F_3

MHco3 (ISE)

MHco3.N2 F_2

MHco3.N2 F_3

Appendix 4.2: Microsatellite Genescan traces for microsatellite marker 26981for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



MHco3 (ISE)

MHco3.N1 F_2

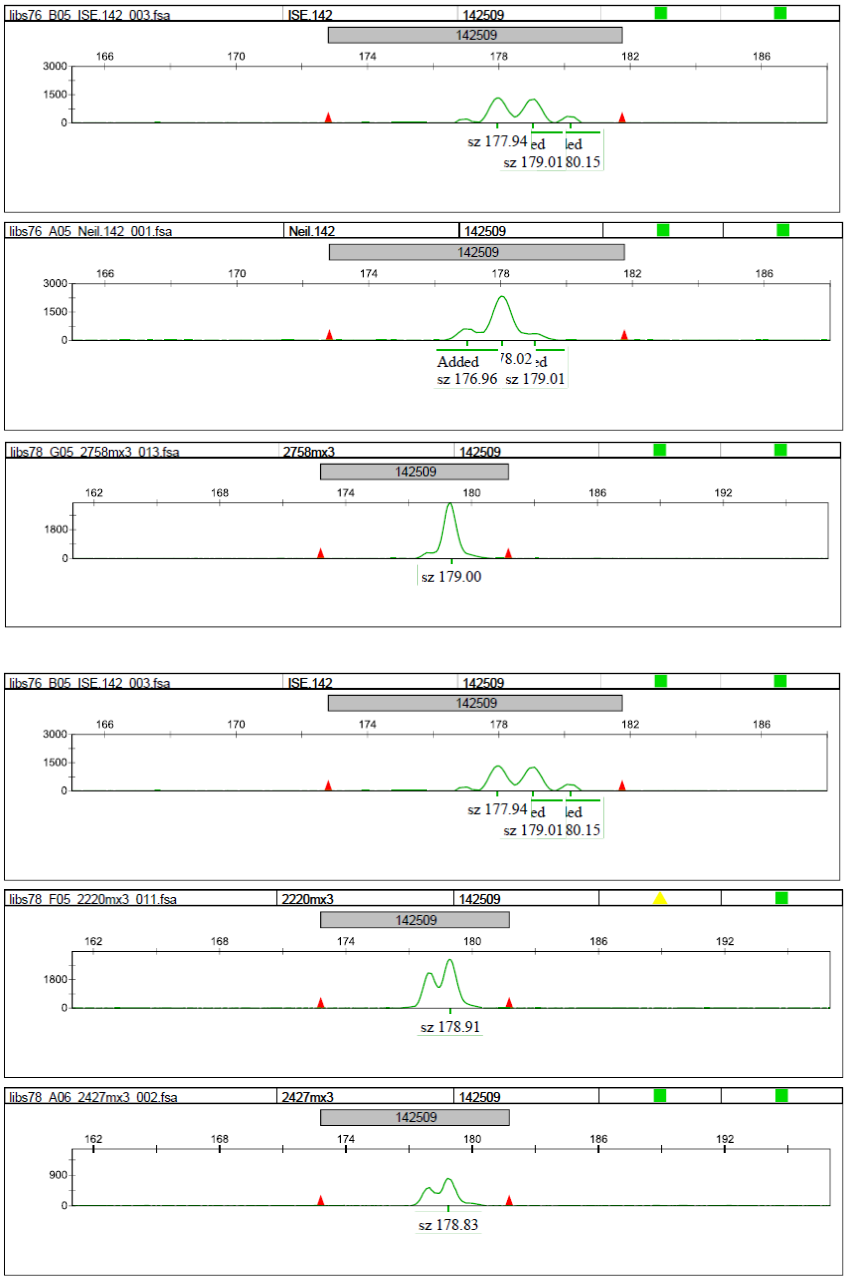
MHco3.N1 F_3

MHco3 (ISE)

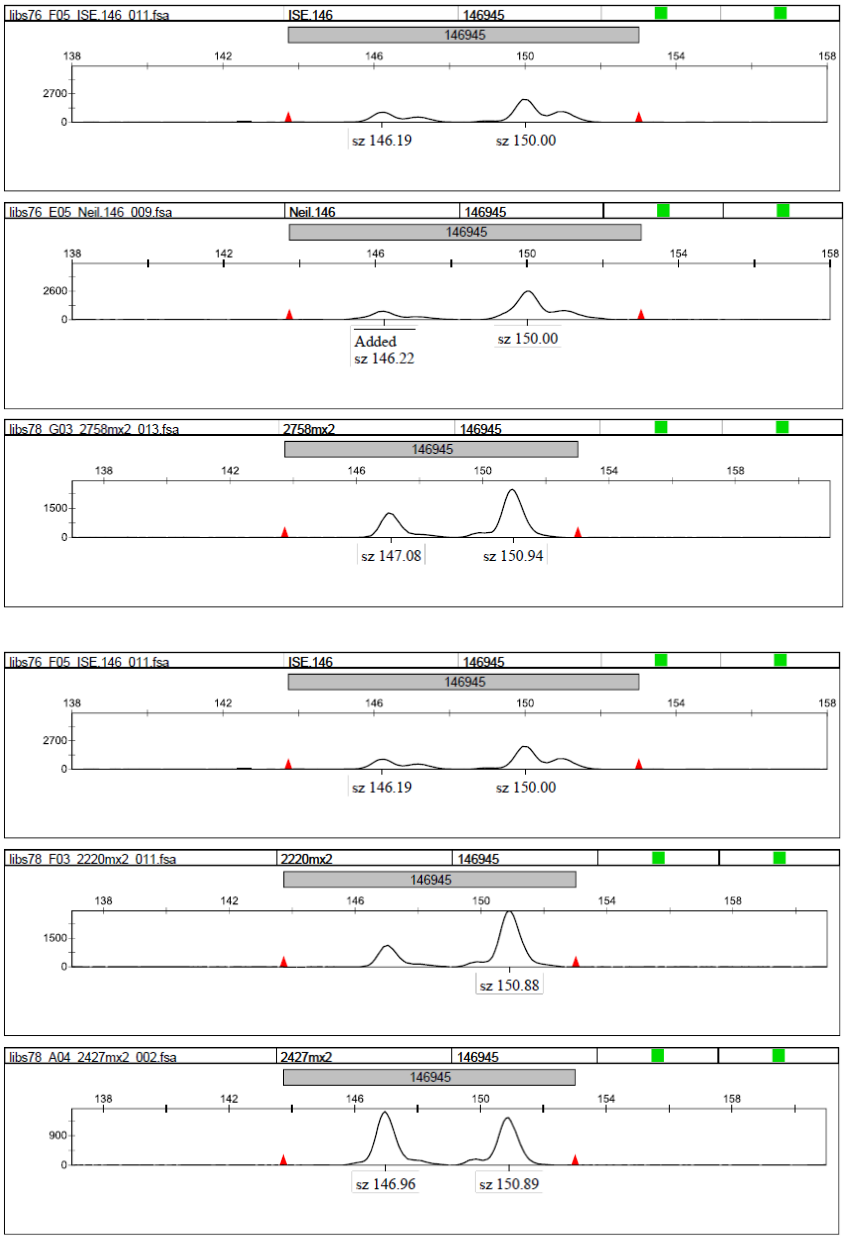
MHco3.N2 F_2

MHco3.N2 F_3

Appendix 4.2: Microsatellite Genescan traces for microsatellite marker 181881 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



Appendix 4.2: Microsatellite Genescan traces for microsatellite marker X142 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



MHco3 (ISE)

MHco3.N1 F_2

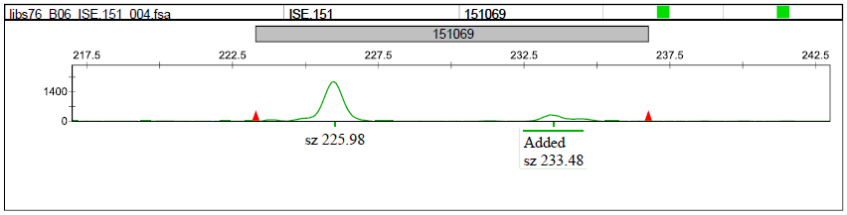
MHco3.N1 F_3

MHco3 (ISE)

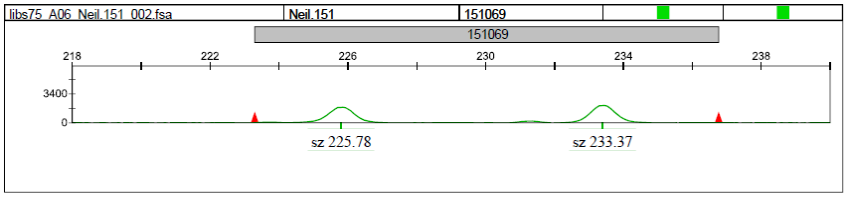
MHco3.N2 F_2

MHco3.N2 F_3

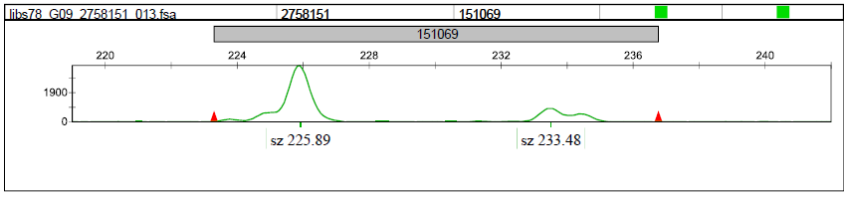
Appendix 4.2: Microsatellite Genescan traces for microsatellite marker X146 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



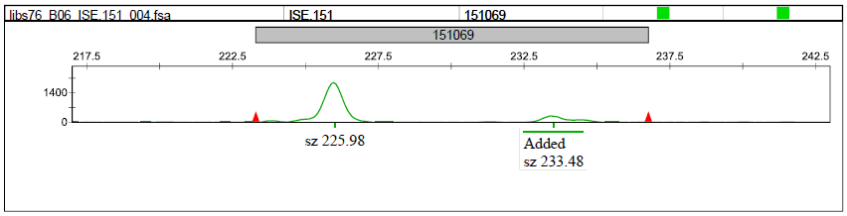
MHco3 (ISE)



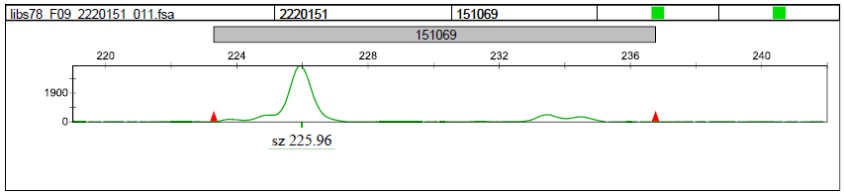
MHco3.N1 F_2



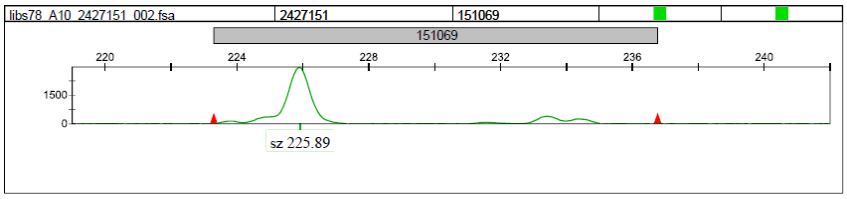
MHco3.N1 F_3



MHco3 (ISE)

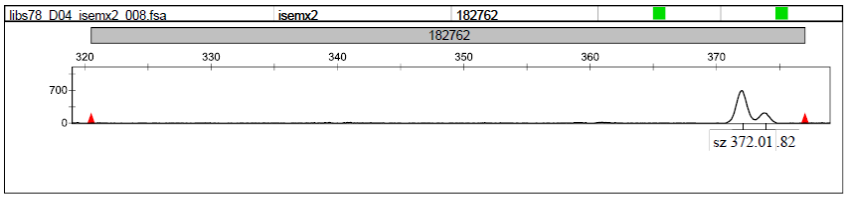


MHco3.N2 F_2

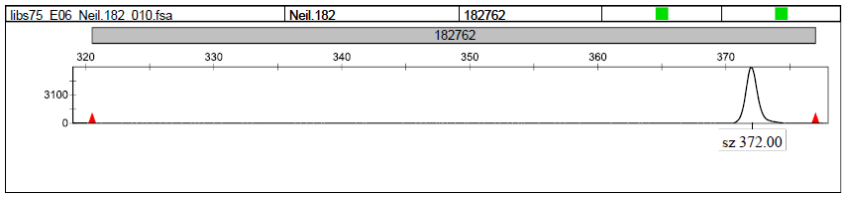


MHco3.N2 F_3

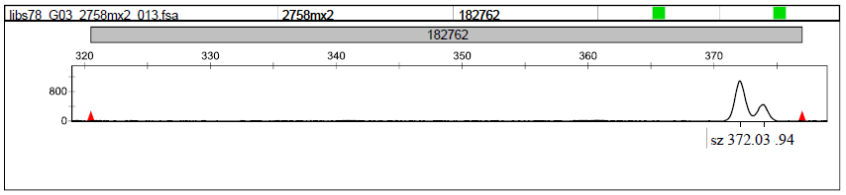
Appendix 4.2: Microsatellite Genescan traces for microsatellite marker X151 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



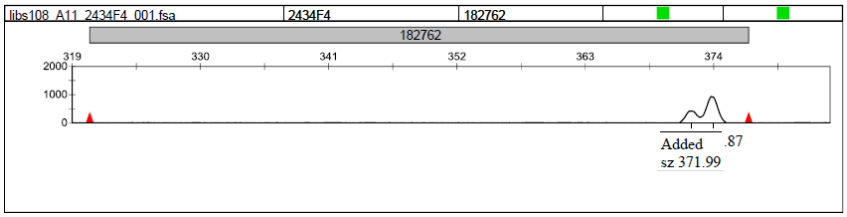
MHco3 (ISE)



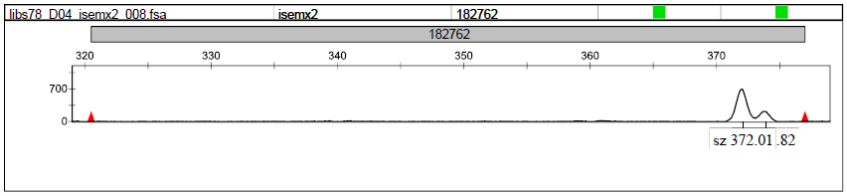
MHco3.N1 F_2



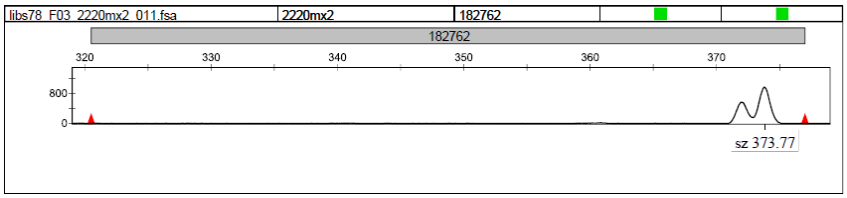
MHco3.N1 F_3



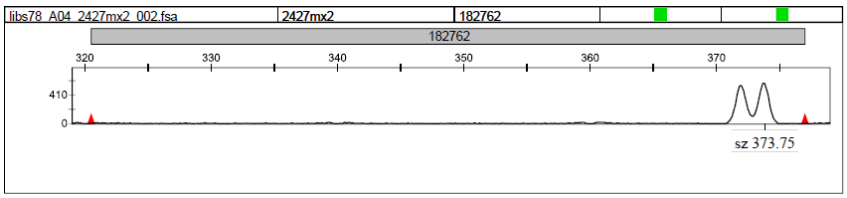
MHco3.N1 F_4



MHco3 (ISE)

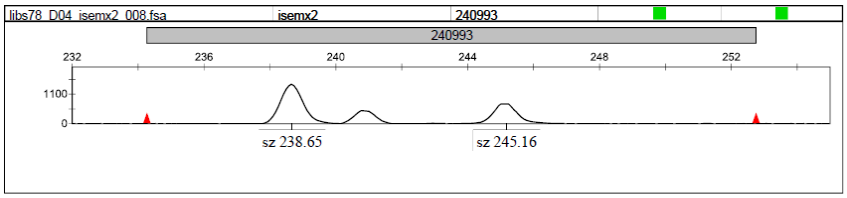


MHco3.N2 F_2

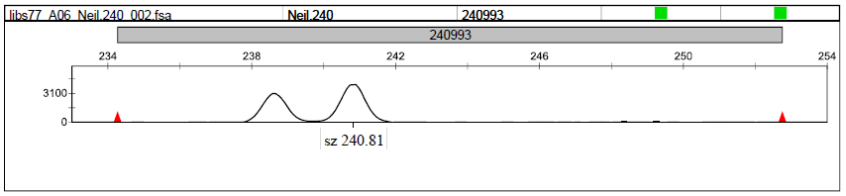


MHco3.N2 F_3

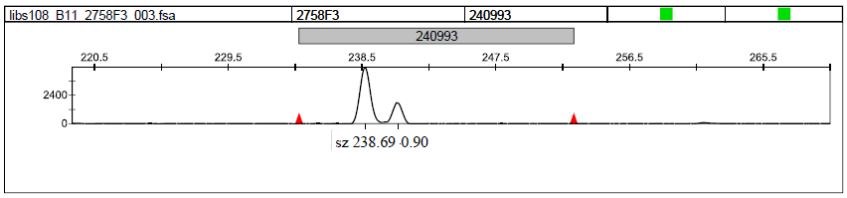
Appendix 4.2: Microsatellite Genescan traces for microsatellite marker X182 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



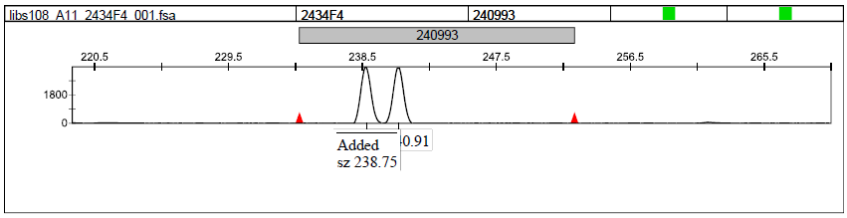
MHco3 (ISE)



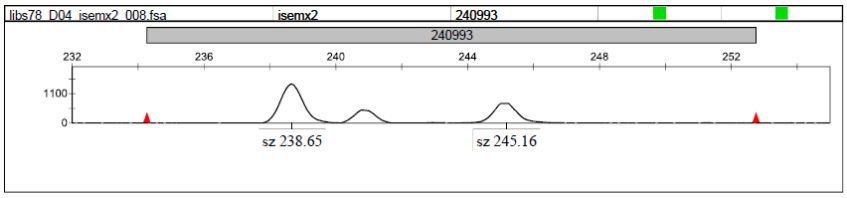
MHco3.N1 F_2



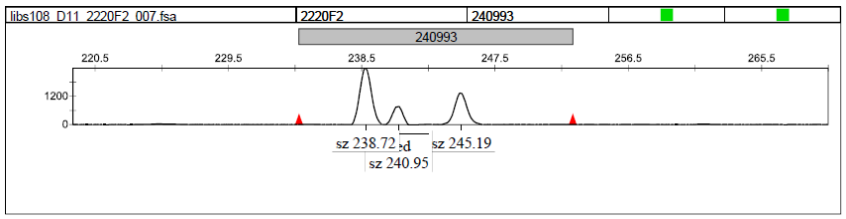
MHco3.N1 F_3



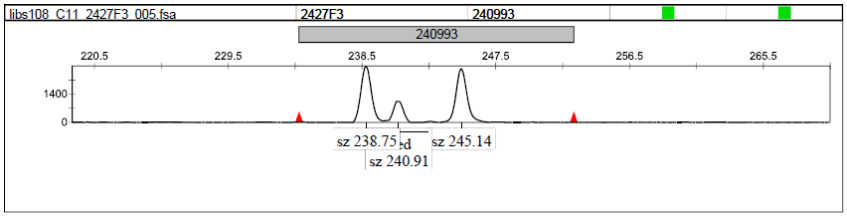
MHco3.N1 F_4



MHco3 (ISE)

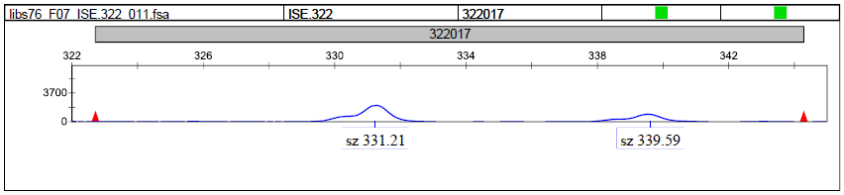


MHco3.N2 F_2

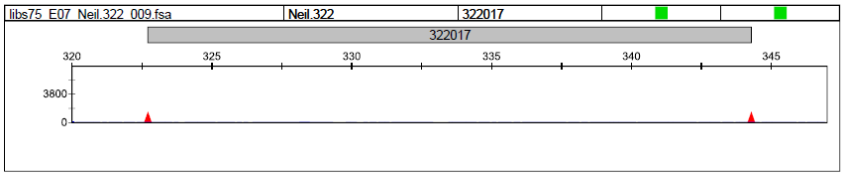


MHco3.N2 F_3

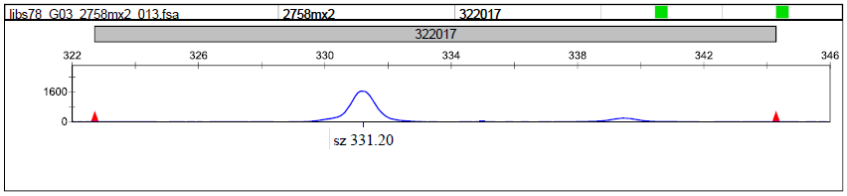
Appendix 4.2: Microsatellite Genescan traces for microsatellite marker X256 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



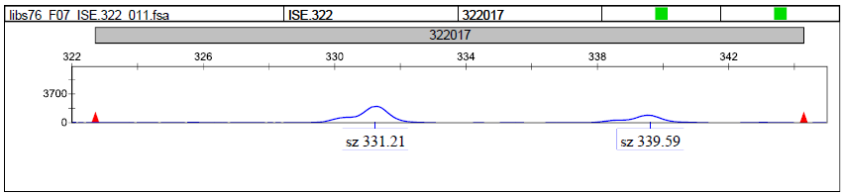
MHco3 (ISE)



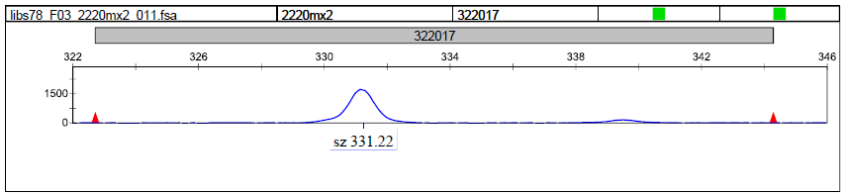
MHco3.N1 F_2



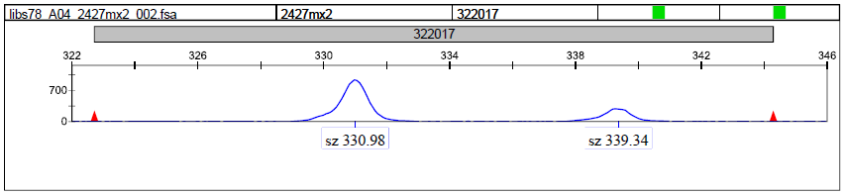
MHco3.N1 F_3



MHco3 (ISE)

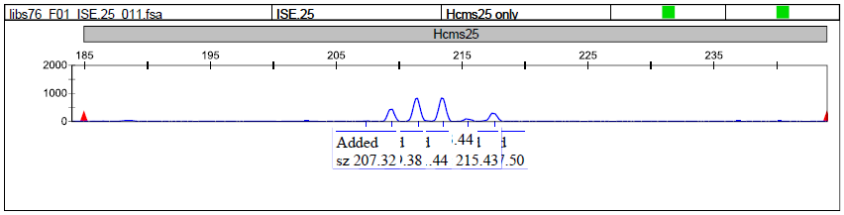


MHco3.N2 F_2

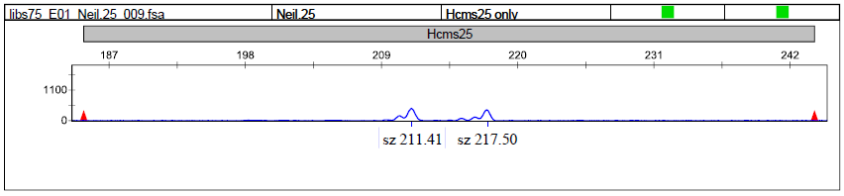


MHco3.N2 F_3

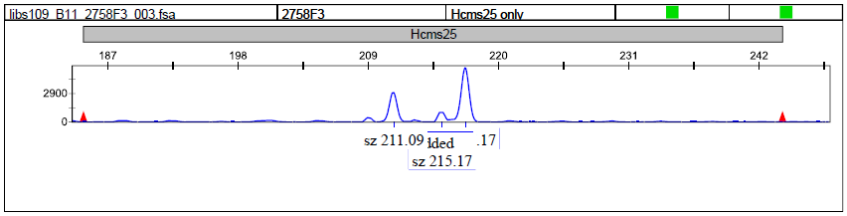
Appendix 4.2: Microsatellite Genescan traces for microsatellite marker X337 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



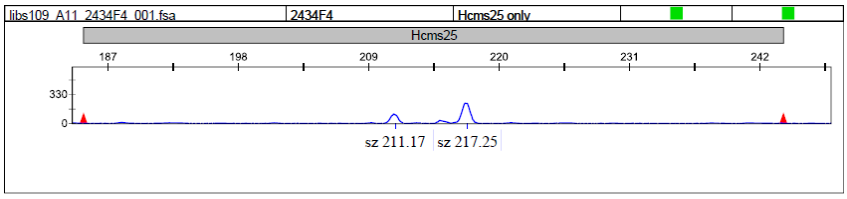
MHco3 (ISE)



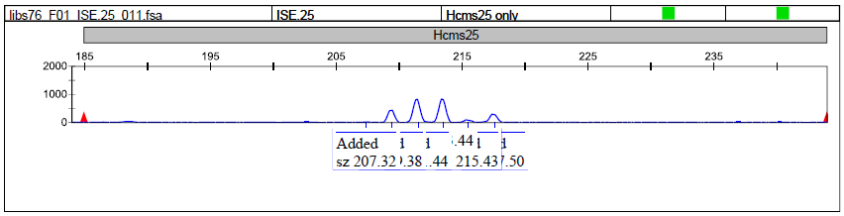
MHco3.N1 F_2



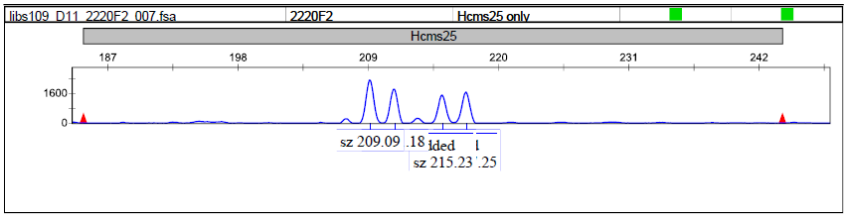
MHco3.N1 F_3



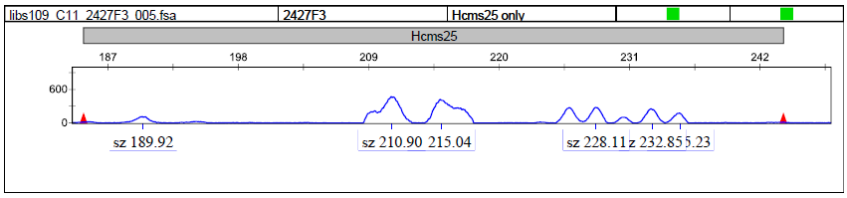
MHco3.N1 F_4



MHco3 (ISE)

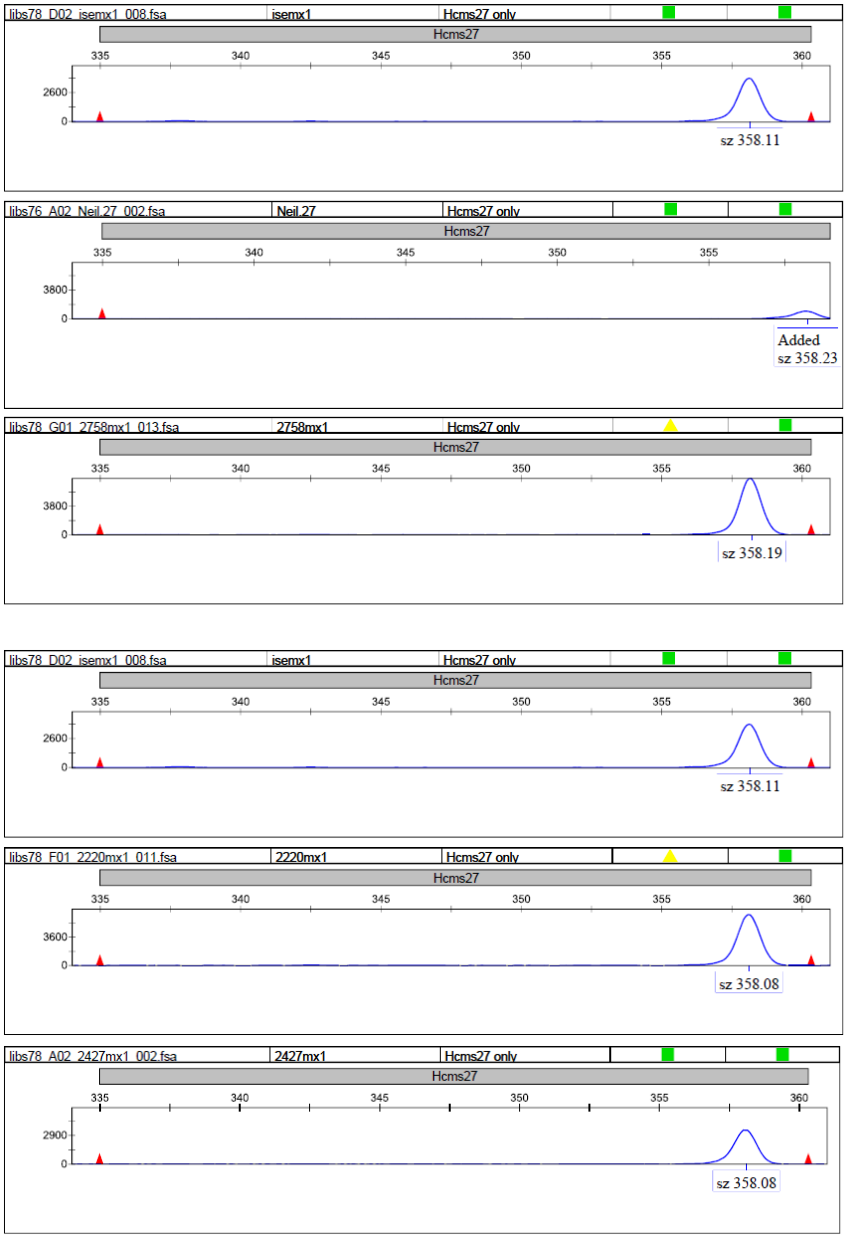


MHco3.N2 F_2

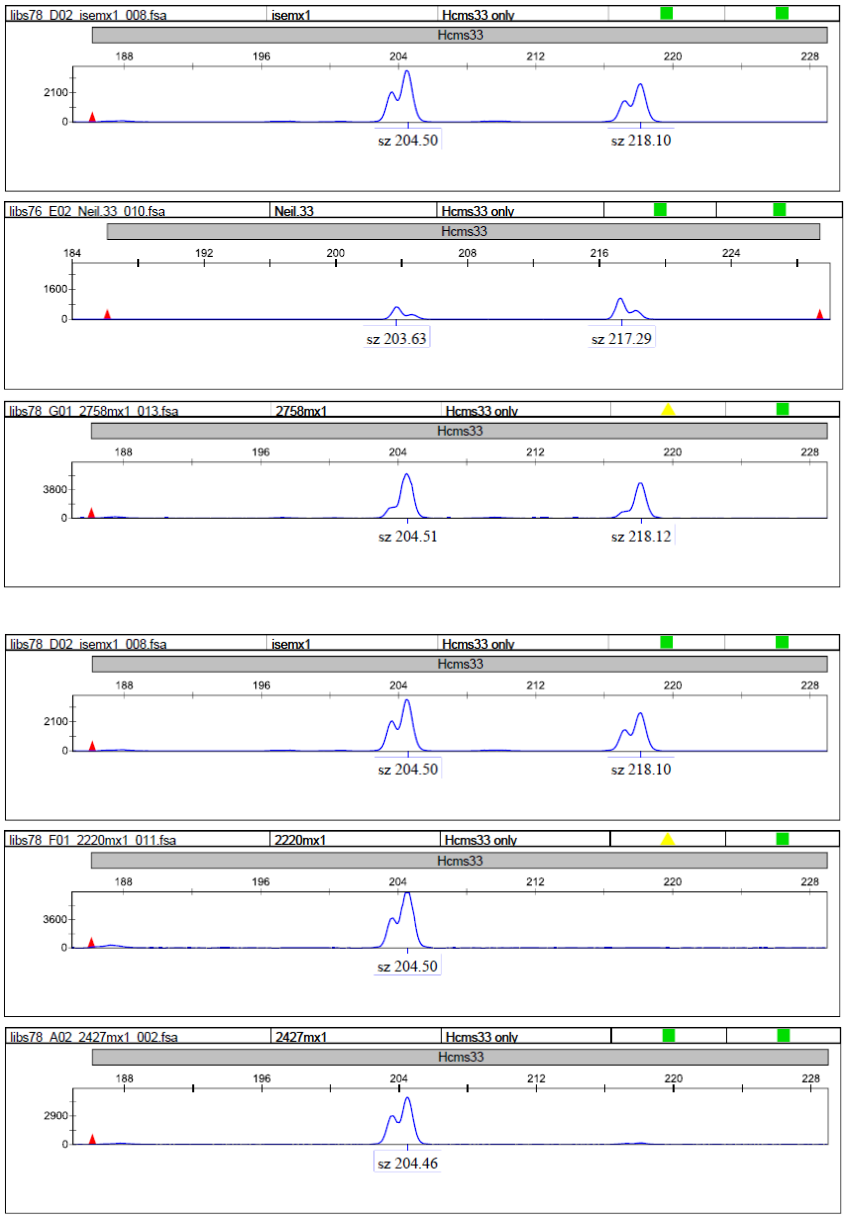


MHco3.N2 F_3

Appendix 4.2: Microsatellite Genescan traces for microsatellite marker Hcms25 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



Appendix 4.2: Microsatellite Genescan traces for microsatellite marker Hcms27 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



MHco3 (ISE)

MHco3.N1 F_2

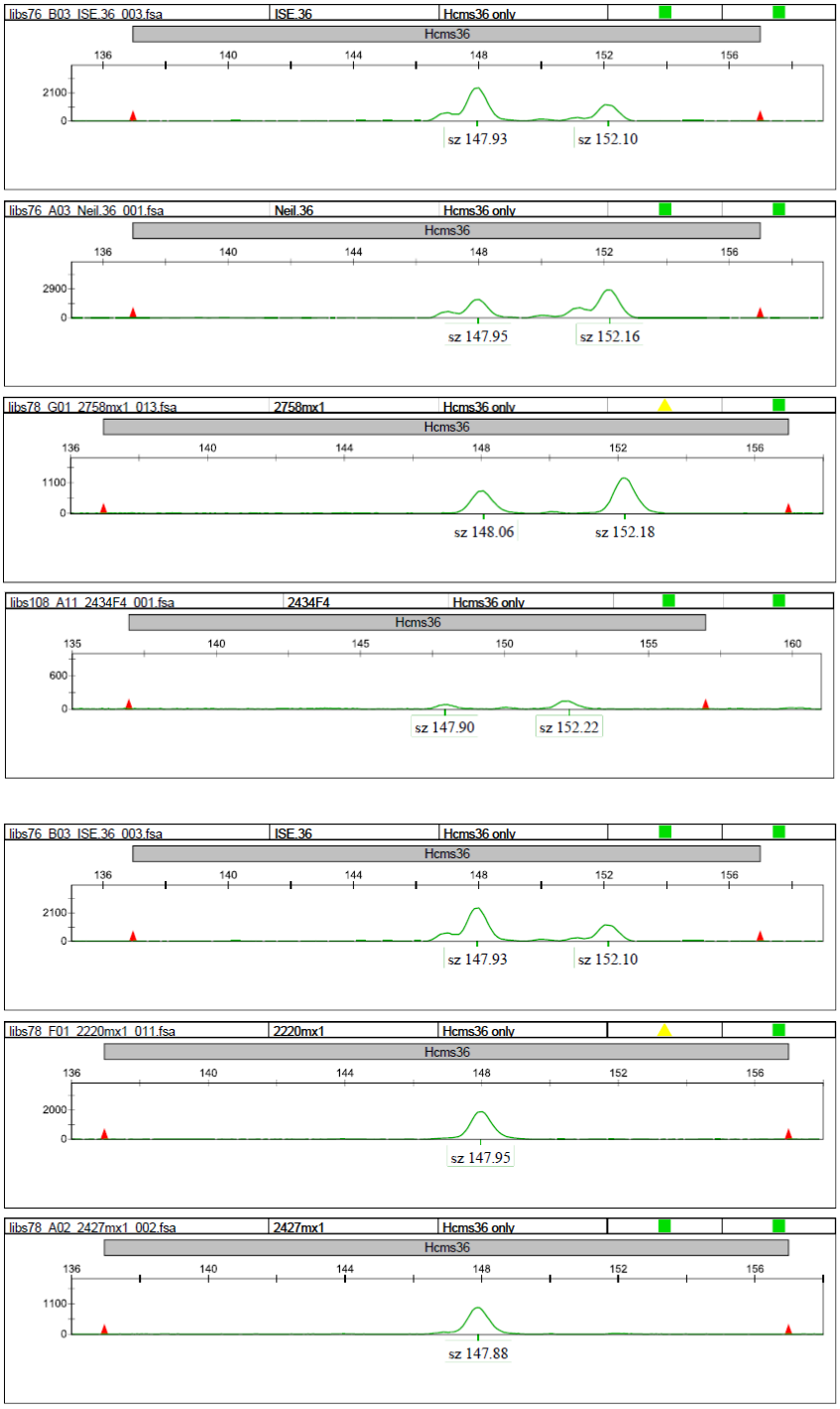
MHco3.N1 F_3

MHco3 (ISE)

MHco3.N2 F_2

MHco3.N2 F_3

Appendix 4.2: Microsatellite Genescan traces for microsatellite marker Hcms33 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



MHco3 (ISE)

MHco3.N1 F_2

MHco3.N1 F_3

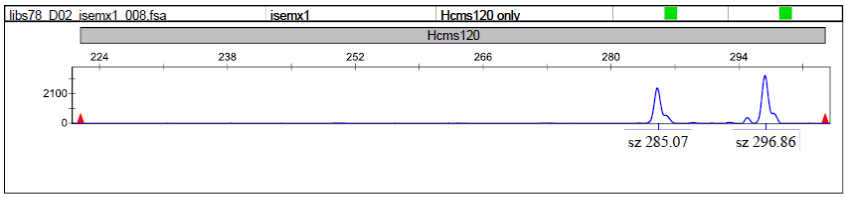
MHco3.N1 F_4

MHco3 (ISE)

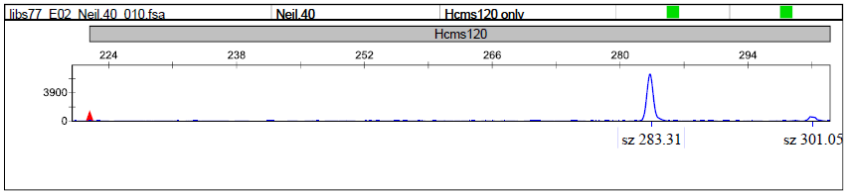
MHco3.N2 F_2

MHco3.N2 F_3

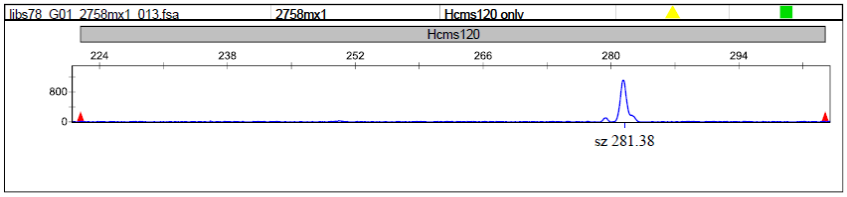
Appendix 4.2: Microsatellite Genescan traces for microsatellite marker Hcms36 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



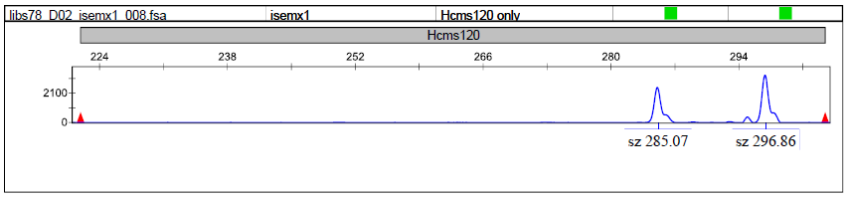
MHco3 (ISE)



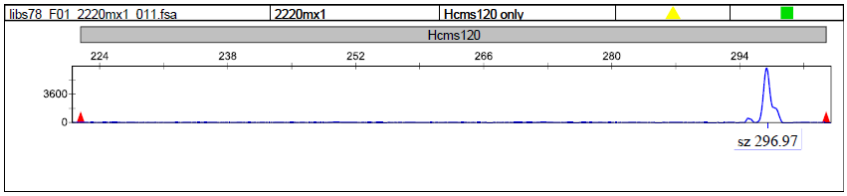
MHco3.N1 F_2



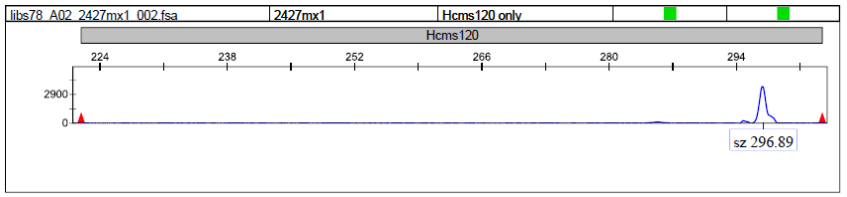
MHco3.N1 F_3



MHco3 (ISE)

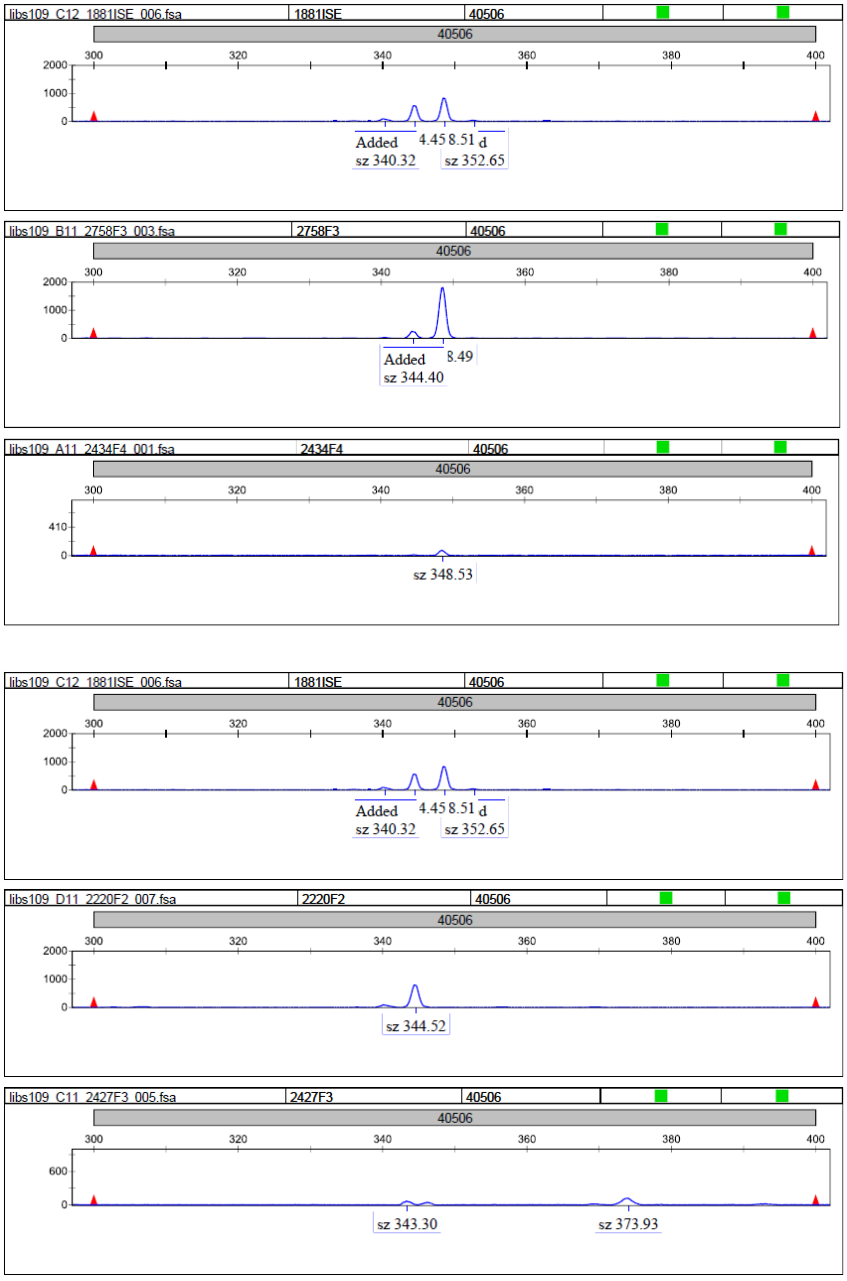


MHco3.N2 F_2



MHco3.N2 F_3

Appendix 4.2: Microsatellite Genescan traces for microsatellite marker Hcms40 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.



Appendix 4.2: Microsatellite Genescan traces for microsatellite marker 40506 for bulk lysates of the parental MHco3 (ISE) strain of *H. contortus* and for the inbred MHco3.N1 and MHco3.N2 lines. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population at each locus are shown.

Appendix 4.3i): Microsatellite genotypes of the founding N1 female parent and individual MHco3.N1 filial nematodes. Data for individual worms where microsatellite markers at several loci were not amplified are not included. Data for marker X142509 were discarded because the alleles were only separated by one base pair and could not be reliably binned. These data show the failure of PCR amplification of microsatellite markers of several individual worms.

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	N1 (♀parent)	Adult ♀	196	196	148	152	261	261	372	372	239	239	211	217
2164A 2	M Hco3.N1.F1	L1	196	196	148	152	261	285	372	374	239	241	211	217
2164A 3	M Hco3.N1.F1	L1	0	0	0	0	261	261	0	0	239	239	217	217
2164A 4	M Hco3.N1.F1	L1	196	196	148	148	261	261	0	0	0	0	211	217
2164A 5	M Hco3.N1.F1	L1	196	196	148	152	261	261	372	372	239	239	211	217
2164A 6	M Hco3.N1.F1	L1	196	196	148	148	261	285	372	372	239	239	211	217
2164A 7	M Hco3.N1.F1	L1	196	196	148	152	261	285	372	372	239	239	211	217
2164A 8	M Hco3.N1.F1	L1	196	196	148	152	261	285	372	372	239	239	217	217
2164A 9	M Hco3.N1.F1	L1	196	196	148	152	261	285	372	372	239	239	211	217
2164A 10	M Hco3.N1.F1	L1	0	0	0	0	261	261	372	372	0	0	217	217
2164A 11	M Hco3.N1.F1	L1	196	196	0	0	261	261	0	0	0	0	217	217
2164A 12	M Hco3.N1.F1	L1	196	196	0	0	0	0	372	372	239	241	211	211
2164A 13	M Hco3.N1.F1	L1	196	196	0	0	261	261	372	372	239	239	217	217
2164A 14	M Hco3.N1.F1	L1	196	196	0	0	0	0	372	372	239	239	211	211
2164A 15	M Hco3.N1.F1	L1	0	0	0	0	261	261	372	374	239	239	217	217
2164A 16	M Hco3.N1.F1	L1	196	196	0	0	261	261	0	0	0	0	211	217
2164A 17	M Hco3.N1.F1	L1	196	196	148	148	261	261	372	372	239	239	211	217
2164A 18	M Hco3.N1.F1	L1	196	196	0	0	261	261	0	0	239	239	211	211
2164A 19	M Hco3.N1.F1	L1	196	196	0	0	261	285	372	374	239	241	217	217
2164A 20	M Hco3.N1.F1	L1	0	0	148	152	261	285	372	374	239	241	211	217
2164A 21	M Hco3.N1.F1	L1	196	196	0	0	261	285	372	372	241	241	211	211
2164A 22	M Hco3.N1.F1	L1	196	196	0	0	261	261	372	372	239	239	211	217
2164A 23	M Hco3.N1.F1	L1	196	196	148	152	261	261	372	372	239	239	211	211
2164A 24	M Hco3.N1.F1	L1	196	212	0	0	261	261	372	372	239	239	211	211
2164A 25	M Hco3.N1.F1	L1	196	196	0	0	261	261	372	372	239	239	211	211
2164A 27	M Hco3.N1.F1	L1	214	214	0	0	261	285	372	372	239	239	211	211
2164A 28	M Hco3.N1.F1	L1	196	196	0	0	261	285	372	374	239	239	211	217
2164A 29	M Hco3.N1.F1	L1	196	196	148	152	261	261	372	374	239	241	0	0
2164A 30	M Hco3.N1.F1	L1	196	196	0	0	261	285	372	372	239	239	211	211
2164A 31	M Hco3.N1.F1	L1	196	196	148	152	261	285	372	374	239	241	211	211
2164A 32	M Hco3.N1.F1	L1	196	196	0	0	261	261	372	372	239	239	217	217
2164A 33	M Hco3.N1.F1	L1	196	196	0	0	261	285	372	372	239	239	211	211
2164A 34	M Hco3.N1.F1	L1	196	196	0	0	261	261	372	372	239	239	217	217
2164A 35	M Hco3.N1.F1	L1	196	196	0	0	261	261	372	372	239	241	217	217
2164A 38	M Hco3.N1.F1	L1	196	196	0	0	261	261	372	372	0	0	217	217
2164A 40	M Hco3.N1.F1	L1	196	196	0	0	261	261	372	374	239	241	211	211
2164A 41	M Hco3.N1.F1	L1	0	0	0	0	261	261	372	372	239	239	217	217
2164A 42	M Hco3.N1.F1	L1	196	196	148	148	261	285	372	372	239	239	211	217
2164A 43	M Hco3.N1.F1	L1	196	196	148	148	261	261	372	374	239	241	211	217
2164A 44	M Hco3.N1.F1	L1	196	196	148	152	261	261	372	374	239	241	211	217
2164A 45	M Hco3.N1.F1	L1	0	0	148	152	261	261	372	374	239	241	211	217
2164A 46	M Hco3.N1.F1	L1	196	196	148	148	261	261	372	374	239	241	211	211

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	N1 (♀parent)	Adult ♀	196	196	148	152	261	261	372	372	239	239	211	217
2274 B	M Hco3.N1.F2	L3	196	196	148	152	285	285	372	374	239	241	0	0
2274 D	M Hco3.N1.F2	L3	0	0	148	152	261	261	372	372	239	239	0	0
2274 F	M Hco3.N1.F2	L3	0	0	148	148	261	261	372	372	239	239	0	0
2274 J	M Hco3.N1.F2	L3	196	196	152	152	261	285	374	374	241	241	211	211
2274 S	M Hco3.N1.F2	L3	0	0	148	152	261	261	374	374	241	241	217	217
2274 a	M Hco3.N1.F2	L3	196	196	0	0	261	261	372	372	0	0	217	217
2274 d	M Hco3.N1.F2	L3	0	0	0	0	261	285	374	374	0	0	211	217
2274 e	M Hco3.N1.F2	L3	196	196	0	0	285	285	374	374	0	0	217	217
2274 m	M Hco3.N1.F2	L3	0	0	0	0	261	285	372	374	0	0	217	217
2274 n	M Hco3.N1.F2	L3	0	0	0	0	285	285	372	372	0	0	217	217
2274 u	M Hco3.N1.F2	L3	196	196	0	0	285	285	372	372	239	239	211	217
2274 v	M Hco3.N1.F2	L3	196	196	0	0	285	285	372	372	239	239	217	217
2758F1	M Hco3.N1.F2	Adult ♀	196	196	152	152	261	261	372	374	239	239	217	217
2758F2	M Hco3.N1.F2	Adult ♀	196	196	152	152	261	285	372	374	239	239	0	0
2758F3	M Hco3.N1.F2	Adult ♀	196	196	0	0	285	285	372	374	239	241	211	217
2758F4	M Hco3.N1.F2	Adult ♀	196	196	0	0	261	261	372	372	239	239	211	217
2758F5	M Hco3.N1.F2	Adult ♀	196	196	0	0	261	261	372	372	239	239	211	217
2758F6	M Hco3.N1.F2	Adult ♀	196	196	148	152	261	285	372	372	241	241	0	0
2758F7	M Hco3.N1.F2	Adult ♀	196	196	148	152	261	261	372	372	239	241	217	217
2758F8	M Hco3.N1.F2	Adult ♀	0	0	148	152	261	261	372	372	239	239	217	217
2758F9	M Hco3.N1.F2	Adult ♀	196	196	148	152	261	261	372	372	239	239	0	0
2758F10	M Hco3.N1.F2	Adult ♀	0	0	0	0	261	261	374	374	239	239	211	217
2758F11	M Hco3.N1.F2	Adult ♀	196	196	0	0	261	285	372	372	239	241	217	217
2758F12	M Hco3.N1.F2	Adult ♀	196	196	152	152	261	285	372	374	239	239	211	217
2758F13	M Hco3.N1.F2	Adult ♀	196	196	0	0	261	285	372	372	239	239	211	217
2758F14	M Hco3.N1.F2	Adult ♀	196	196	152	152	261	285	372	374	239	241	217	217
2758F15	M Hco3.N1.F2	Adult ♀	196	196	0	0	261	285	372	372	239	239	217	217
2758F16	M Hco3.N1.F2	Adult ♀	196	196	152	152	261	285	372	372	239	239	217	217
2758F18	M Hco3.N1.F2	Adult ♀	0	0	0	0	261	285	372	374	239	239	0	0
2758F19	M Hco3.N1.F2	Adult ♀	196	196	0	0	261	261	372	372	239	239	217	217
2758F20	M Hco3.N1.F2	Adult ♀	196	196	0	0	261	285	372	374	239	239	211	217
2758F21	M Hco3.N1.F2	Adult ♀	0	0	0	0	261	261	372	372	239	239	217	217
2758F22	M Hco3.N1.F2	Adult ♀	0	0	0	0	261	261	372	374	239	241	217	217
2758F23	M Hco3.N1.F2	Adult ♀	0	0	0	0	261	285	372	372	239	239	217	217
2758F24	M Hco3.N1.F2	Adult ♀	0	0	0	0	261	285	372	372	239	239	211	217
2758F25	M Hco3.N1.F2	Adult ♀	0	0	152	152	261	285	372	374	239	241	217	217
2758F26	M Hco3.N1.F2	Adult ♀	0	0	0	0	285	285	372	372	239	241	217	217
2758M1	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	285	374	374	241	241	211	211
2758M2	M Hco3.N1.F2	Adult ♂	196	196	0	0	261	261	372	372	239	239	211	217
2758M6	M Hco3.N1.F2	Adult ♂	0	0	148	152	261	285	372	372	239	239	211	217
2758M7	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	261	374	374	0	0	211	217
2758M8	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	261	374	374	241	241	217	217
2758M9	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	261	374	374	239	241	211	217
2758M10	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	261	374	374	241	241	211	217
2758M11	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	285	0	0	241	241	217	217
2758M12	M Hco3.N1.F2	Adult ♂	0	0	148	152	285	285	372	372	239	241	217	217
2758M14	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	285	374	374	241	241	211	217
2758M15	M Hco3.N1.F2	Adult ♂	0	0	0	0	285	285	372	372	239	239	211	217
2758M16	M Hco3.N1.F2	Adult ♂	0	0	148	148	261	261	372	374	241	241	211	217
2758M17	M Hco3.N1.F2	Adult ♂	0	0	148	152	261	285	374	374	241	241	0	0
2758M18	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	285	372	372	239	239	217	217
2758M19	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	261	372	372	239	239	211	217
2758M20	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	285	374	374	241	241	211	217
2758M21	M Hco3.N1.F2	Adult ♂	0	0	0	0	0	0	372	372	239	239	217	217
2758M22	M Hco3.N1.F2	Adult ♂	0	0	148	148	261	261	372	372	239	241	211	217
2758M23	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	285	374	374	241	241	217	217
2758M24	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	261	372	372	239	241	211	217
2758M25	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	285	372	372	239	239	211	211
2758M26	M Hco3.N1.F2	Adult ♂	0	0	0	0	261	285	374	374	239	239	211	217

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	N1 (♀parent)	Adult ♀	196	196	148	152	261	261	372	372	239	239	211	217
2758 D	M Hco3.N1.F3	L3	0	0	148	152	261	261	374	374	241	241	0	0
2758 E	M Hco3.N1.F3	L3	196	196	148	152	261	261	372	372	239	239	211	217
2758 F	M Hco3.N1.F3	L3	0	0	152	152	261	261	372	374	239	241	213	217
2758 M	M Hco3.N1.F3	L3	0	0	148	152	261	261	374	374	241	241	211	211
2758 N	M Hco3.N1.F3	L3	0	0	152	152	261	261	372	372	239	239	0	0
2758 R	M Hco3.N1.F3	L3	0	0	148	148	261	261	372	372	239	239	0	0
2758 a	M Hco3.N1.F3	L3	196	196	0	0	261	285	372	372	0	0	211	217
2758 b	M Hco3.N1.F3	L3	196	196	0	0	261	261	372	374	0	0	211	217
2758 c	M Hco3.N1.F3	L3	196	196	0	0	261	285	372	372	0	0	211	217
2758 d	M Hco3.N1.F3	L3	0	0	0	0	261	261	372	372	0	0	211	217
2758 e	M Hco3.N1.F3	L3	196	196	0	0	261	261	372	374	0	0	217	217
2758 f	M Hco3.N1.F3	L3	0	0	0	0	261	285	372	372	0	0	217	217
2758 h	M Hco3.N1.F3	L3	196	196	0	0	285	285	372	372	0	0	217	217
2758 i	M Hco3.N1.F3	L3	196	196	0	0	261	261	372	374	0	0	211	217
2758 j	M Hco3.N1.F3	L3	196	196	0	0	261	285	372	372	0	0	211	211
2758 k	M Hco3.N1.F3	L3	196	196	0	0	285	285	374	374	0	0	217	217
2758 l	M Hco3.N1.F3	L3	196	196	0	0	261	285	374	374	0	0	211	217
2758 m	M Hco3.N1.F3	L3	196	196	0	0	261	285	372	374	0	0	217	217
2758 n	M Hco3.N1.F3	L3	0	0	0	0	261	261	372	372	0	0	211	217
2758 o	M Hco3.N1.F3	L3	196	196	0	0	261	285	372	372	0	0	211	217
2758 p	M Hco3.N1.F3	L3	196	196	0	0	261	261	372	374	0	0	211	217
2758 q	M Hco3.N1.F3	L3	196	196	0	0	261	285	372	372	0	0	211	217
2758 r	M Hco3.N1.F3	L3	196	196	0	0	261	261	374	374	0	0	211	217
2758 s	M Hco3.N1.F3	L3	196	196	0	0	261	261	372	372	0	0	211	217
2758 t	M Hco3.N1.F3	L3	196	196	0	0	261	261	372	374	0	0	217	217
2758 u	M Hco3.N1.F3	L3	196	196	0	0	0	0	0	0	0	0	211	217
2758 v	M Hco3.N1.F3	L3	196	196	0	0	285	285	0	0	0	0	217	217
2758 w	M Hco3.N1.F3	L3	196	196	0	0	261	261	372	372	239	239	211	217
2758 x	M Hco3.N1.F3	L3	196	196	0	0	261	261	374	374	0	0	211	217
2758E.2	M Hco3.N1.F3	L3	196	196	0	0	261	261	372	372	239	239	211	217
2434 B	M Hco3.N1.F4	L3	196	196	148	152	261	261	372	374	239	241	211	211
2434 C	M Hco3.N1.F4	L3	196	196	0	0	0	0	374	374	239	239	217	217
2434 D	M Hco3.N1.F4	L3	196	196	148	152	261	261	372	374	239	241	211	217
2434 E	M Hco3.N1.F4	L3	196	196	152	152	261	261	372	372	239	239	217	217
2434 F	M Hco3.N1.F4	L3	196	196	152	152	261	261	372	374	239	241	217	217
2434 G	M Hco3.N1.F4	L3	0	0	148	148	261	261	372	374	239	241	0	0
2434 H	M Hco3.N1.F4	L3	0	0	152	152	261	261	372	372	239	239	0	0
2434 I	M Hco3.N1.F4	L3	0	0	148	152	261	261	372	374	239	241	0	0
2434 J	M Hco3.N1.F4	L3	196	196	148	152	261	261	372	374	239	241	217	217
2434 K	M Hco3.N1.F4	L3	196	196	152	152	261	285	372	374	239	241	217	217
2434 L	M Hco3.N1.F4	L3	196	196	148	152	261	261	374	374	241	241	211	217
2434 R	M Hco3.N1.F4	L3	0	0	148	152	261	285	372	374	239	241	0	0
2434 S	M Hco3.N1.F4	L3	0	0	148	152	261	261	372	374	239	241	0	0
2434 J2	M Hco3.N1.F4	L3	196	212	0	0	261	261	0	0	0	0	217	217

Appendix 4.3ii): Microsatellite genotypes of the founding N2 female parent and individual MHco3.N2 filial nematodes. Data for individual worms where microsatellite markers at several loci were not amplified are not included. Data for marker X142509 were discarded because the alleles were only separated by one base pair and could not be reliably binned. These data show the failure of PCR amplification of microsatellite markers of several individual worms.

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2144G Fem	N2 (♀parent)	Adult ♀	232	232	148	148	261	287	372	374	241	245	211	217
2144G 1	M Hco3.N2.F1	L1	232	232	148	148	261	285	374	374	239	241	209	211
2144G 2	M Hco3.N2.F1	L1	0	0	148	148	287	287	372	374	239	239	211	215
2144G 12	M Hco3.N2.F1	L1	232	232	148	148	261	285	372	374	241	245	209	211
2144G 13	M Hco3.N2.F1	L1	232	232	148	148	261	287	372	374	241	245	209	217
2144G 15	M Hco3.N2.F1	L1	232	232	148	148	287	287	372	374	239	245	217	217
2144G 16	M Hco3.N2.F1	L1	232	232	148	148	261	285	374	374	241	241	217	217
2144G 21	M Hco3.N2.F1	L1	0	0	148	148	287	287	374	374	239	241	0	0
2144G 23	M Hco3.N2.F1	L1	232	232	148	148	287	287	372	374	239	245	0	0
2144G 26	M Hco3.N2.F1	L1	0	0	0	0	261	287	372	372	241	245	211	217
2144G 28	M Hco3.N2.F1	L1	232	232	0	0	261	287	0	0	241	245	0	0
2144G 29	M Hco3.N2.F1	L1	232	232	0	0	261	287	374	374	245	245	0	0
2144G 32	M Hco3.N2.F1	L1	0	0	0	0	0	0	0	0	241	245	209	217
2220F1	M Hco3.N2.F1	Adult ♀	232	232	148	148	261	285	372	374	239	245	209	217
2220F2	M Hco3.N2.F1	Adult ♀	232	232	148	148	287	287	372	374	239	245	215	217
2220F3	M Hco3.N2.F1	Adult ♀	232	232	148	148	261	285	372	374	239	245	215	217
2220F4	M Hco3.N2.F1	Adult ♀	232	232	0	0	261	285	374	374	239	241	211	211
2220F5	M Hco3.N2.F1	Adult ♀	232	232	148	148	287	287	374	374	239	241	215	217
2220F6	M Hco3.N2.F1	Adult ♀	196	196	148	152	261	261	372	374	239	241	211	217
2220F7	M Hco3.N2.F1	Adult ♀	0	0	0	0	287	287	374	374	239	241	209	217
2220M1	M Hco3.N2.F1	Adult ♂	232	232	0	0	287	287	374	374	241	241	209	211
2220M3	M Hco3.N2.F1	Adult ♂	0	0	0	0	285	287	374	374	241	241	209	217

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2144G Fem	N2 (♀parent)	Adult ♀	232	232	148	148	261	287	372	374	241	245	211	217
2220 A	M Hco3.N2.F2	L3	0	0	148	148	261	287	374	374	239	241	0	0
2220 C	M Hco3.N2.F2	L3	0	0	148	148	285	285	374	374	239	239	0	0
2220 D	M Hco3.N2.F2	L3	0	0	148	148	287	287	374	374	241	241	0	0
2220 E	M Hco3.N2.F2	L3	0	0	148	148	285	287	374	374	241	241	0	0
2220 F	M Hco3.N2.F2	L3	0	0	148	148	261	287	372	372	245	245	0	0
2220 G	M Hco3.N2.F2	L3	0	0	148	148	261	287	372	374	241	245	0	0
2220 H	M Hco3.N2.F2	L3	0	0	148	148	285	285	372	374	241	245	0	0
2220 J	M Hco3.N2.F2	L3	196	196	148	148	287	287	374	374	241	241	0	0
2220 K	M Hco3.N2.F2	L3	196	196	148	148	285	285	372	374	239	245	0	0
2220 L	M Hco3.N2.F2	L3	0	0	148	148	285	285	374	374	241	241	0	0
2220 M	M Hco3.N2.F2	L3	0	0	148	148	261	285	372	374	239	245	0	0
2220 N	M Hco3.N2.F2	L3	0	0	148	148	285	285	372	372	245	245	0	0
2220 O	M Hco3.N2.F2	L3	0	0	148	148	261	287	372	374	239	245	0	0
2220 a	M Hco3.N2.F2	L3	0	0	0	0	285	285	372	374	0	0	211	217
2220 b	M Hco3.N2.F2	L3	232	232	0	0	285	287	372	372	245	245	215	215
2220 c	M Hco3.N2.F2	L3	0	0	0	0	261	261	374	374	0	0	217	217
2220 d	M Hco3.N2.F2	L3	232	232	0	0	287	287	372	372	245	245	209	217
2220 e	M Hco3.N2.F2	L3	232	232	0	0	285	287	372	374	0	0	211	215
2220 f	M Hco3.N2.F2	L3	232	232	0	0	261	287	372	372	0	0	211	217
2220 g	M Hco3.N2.F2	L3	232	232	0	0	285	287	374	374	0	0	209	215
2220 h	M Hco3.N2.F2	L3	232	232	0	0	0	0	374	374	0	0	217	217
2220 i	M Hco3.N2.F2	L3	232	232	0	0	285	285	372	372	0	0	215	217
2220 j	M Hco3.N2.F2	L3	232	232	0	0	287	287	372	372	0	0	209	217
2220 l	M Hco3.N2.F2	L3	232	232	0	0	261	261	372	374	0	0	209	209
2220 m	M Hco3.N2.F2	L3	196	232	0	0	285	285	374	374	0	0	211	215
2220 n	M Hco3.N2.F2	L3	232	232	0	0	287	287	372	372	0	0	0	0
2220 o	M Hco3.N2.F2	L3	232	232	0	0	285	285	372	374	0	0	209	217
2220 p	M Hco3.N2.F2	L3	232	232	0	0	261	287	374	374	0	0	211	217
2220 r	M Hco3.N2.F2	L3	232	232	0	0	0	0	372	372	0	0	209	211
2220 t	M Hco3.N2.F2	L3	196	196	0	0	0	0	0	0	0	0	217	217
2220 u	M Hco3.N2.F2	L3	196	232	0	0	285	285	374	374	0	0	209	215
2220 v	M Hco3.N2.F2	L3	232	232	0	0	285	287	372	372	0	0	209	209
2220 w	M Hco3.N2.F2	L3	232	232	0	0	261	287	374	374	0	0	209	217
2220Q2	M Hco3.N2.F2	L3	240	240	148	148	285	285	374	374	241	245	0	0

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2144G Fem	N2 (♀parent)	Adult ♀	232	232	148	148	261	287	372	374	241	245	211	217
2427 A	M Hco3.N2.F3	L3	0	0	148	148	285	287	374	374	241	241	0	0
2427 C	M Hco3.N2.F3	L3	0	0	148	148	261	285	372	374	239	245	0	0
2427 D	M Hco3.N2.F3	L3	232	232	148	148	285	285	374	374	241	241	215	215
2427 G	M Hco3.N2.F3	L3	0	0	148	148	287	287	372	374	239	239	0	0
2427 K	M Hco3.N2.F3	L3	0	0	148	148	285	285	374	374	239	245	0	0
2427 L	M Hco3.N2.F3	L3	232	232	148	148	285	285	372	374	239	239	211	211
2427 M	M Hco3.N2.F3	L3	0	0	148	148	287	287	374	374	239	241	217	217
2427 O	M Hco3.N2.F3	L3	0	0	148	148	287	287	374	374	239	239	0	0
2427 P	M Hco3.N2.F3	L3	0	0	148	148	287	287	374	374	239	239	0	0
2427 R	M Hco3.N2.F3	L3	0	0	148	148	285	287	372	372	241	245	0	0
2427 b	M Hco3.N2.F3	L3	196	196	0	0	285	285	374	374	0	0	211	217
2427 c	M Hco3.N2.F3	L3	0	0	0	0	261	285	374	374	0	0	217	217
2427 f	M Hco3.N2.F3	L3	0	0	0	0	285	285	372	374	0	0	211	211
2427 g	M Hco3.N2.F3	L3	0	0	0	0	261	285	372	372	0	0	211	217
2427 j	M Hco3.N2.F3	L3	0	0	0	0	261	261	372	372	0	0	211	217
2427 k	M Hco3.N2.F3	L3	232	232	0	0	285	287	372	374	0	0	211	215
2427 l	M Hco3.N2.F3	L3	232	232	0	0	261	285	374	374	0	0	211	215
2427 m	M Hco3.N2.F3	L3	196	196	0	0	0	0	0	0	239	239	211	217
2427 q	M Hco3.N2.F3	L3	232	232	0	0	287	287	372	372	0	0	211	217
2427 t	M Hco3.N2.F3	L3	232	232	0	0	285	285	372	372	245	245	215	215
2427 u	M Hco3.N2.F3	L3	232	232	0	0	261	261	0	0	0	0	209	209
2427 x	M Hco3.N2.F3	L3	232	232	0	0	261	287	374	374	0	0	215	217
2427D2	M Hco3.N2.F3	L3	232	232	0	0	287	287	374	374	0	0	215	215

Appendix 4.3iii): Microsatellite genotypes of the founding N1 and N2 female parents and individual MHco3 nematodes. Data for individual worms where microsatellite markers at several loci were not amplified are not included. Data for marker X142509 were discarded because the alleles were only separated by one base pair and could not be reliably binned. These data show the failure of PCR amplification of microsatellite markers of several individual worms.

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	**N1 (♀parent)**	**Adult ♀**	**196**	**196**	**148**	**152**	**261**	**261**	**372**	**372**	**239**	**239**	**211**	**217**
2144G Fem	**N2 (♀parent)**	**Adult ♀**	**232**	**232**	**148**	**148**	**261**	**287**	**372**	**374**	**241**	**245**	**211**	**217**
0	M Hco3	L3	192	232	148	152	261	261	372	374	241	245	209	211
1	M Hco3	L3	232	240	148	148	285	285	372	374	239	241	211	211
2	M Hco3	L3	232	240	148	152	261	285	372	374	239	241	211	213
3	M Hco3	L3	192	232	148	148	261	261	372	372	239	239	213	213
4	M Hco3	L3	192	196	152	152	261	261	372	374	239	241	211	211
5	M Hco3	L3	192	232	148	152	285	285	372	374	239	241	213	213
6	M Hco3	L3	0	0	0	0	261	261	372	374	241	245	213	213
7	M Hco3	L3	196	196	148	152	285	285	372	374	239	241	211	217
8	M Hco3	L3	192	232	148	152	261	285	372	372	239	239	211	213
9	M Hco3	L3	192	232	148	152	285	287	372	372	239	239	211	211
10	M Hco3	L3	192	240	148	152	261	287	372	374	239	239	209	213
11	M Hco3	L3	192	192	148	148	285	285	372	374	239	241	213	215
12	M Hco3	L3	196	196	148	152	261	285	374	374	241	241	217	217
13	M Hco3	L3	192	232	148	152	261	285	374	374	241	241	213	213
14	M Hco3	L3	240	240	148	152	261	287	372	374	239	241	213	217
17	M Hco3	L3	192	240	148	152	261	285	372	372	239	239	215	215
18	M Hco3	L3	196	196	148	148	261	285	372	372	239	245	213	213
19	M Hco3	L3	192	240	152	152	261	285	372	372	239	239	213	213
20	M Hco3	L3	192	232	148	152	261	261	372	372	239	239	213	213
21	M Hco3	L3	192	240	148	148	261	261	372	372	239	239	213	213
22	M Hco3	L3	192	232	148	152	261	261	372	374	239	241	211	213
23	M Hco3	L3	232	232	148	148	261	285	372	372	239	239	217	217
24	M Hco3	L3	192	232	148	148	261	285	372	374	239	241	211	213
25	M Hco3	L3	232	232	148	152	261	285	372	372	239	239	0	0
26	M Hco3	L3	232	240	148	148	261	285	372	374	239	241	211	211
27	M Hco3	L3	232	232	148	152	261	285	372	372	239	239	211	217
28	M Hco3	L3	232	240	148	148	261	285	372	374	239	241	213	213
29	M Hco3	L3	240	240	148	152	261	285	374	374	241	241	209	213

Appendix 4.4i): Microsatellite genotypes of the founding N1 female parent and individual MHco3.N1 filial nematodes. Data are shown for individual worms where one or less of six microsatellites were not amplified.

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	N1 (♀parent)	Adult ♀	196	196	148	152	262	262	372	372	240	240	211	217
2164A 2	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	374	240	242	211	217
2164A 5	M Hco3.N1.F1	L1	196	196	148	152	262	262	372	372	240	240	211	217
2164A 6	M Hco3.N1.F1	L1	196	196	148	148	262	285	372	372	240	240	211	217
2164A 7	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	372	240	240	211	217
2164A 8	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	372	240	240	217	217
2164A 9	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	372	240	240	211	217
2164A 17	M Hco3.N1.F1	L1	196	196	148	148	262	262	372	372	240	240	211	217
2164A 19	M Hco3.N1.F1	L1	196	196	0	0	262	285	372	374	240	242	217	217
2164A 20	M Hco3.N1.F1	L1	0	0	148	152	262	285	372	374	240	242	211	217
2164A 21	M Hco3.N1.F1	L1	196	196	0	0	262	285	372	372	242	242	211	211
2164A 22	M Hco3.N1.F1	L1	196	196	0	0	262	262	372	372	240	240	211	217
2164A 23	M Hco3.N1.F1	L1	196	196	148	152	262	262	372	372	240	240	211	211
2164A 24	M Hco3.N1.F1	L1	196	212	0	0	262	262	372	372	240	240	211	211
2164A 25	M Hco3.N1.F1	L1	196	196	0	0	262	262	372	372	240	240	211	211
2164A 27	M Hco3.N1.F1	L1	214	214	0	0	262	285	372	372	240	240	211	211
2164A 28	M Hco3.N1.F1	L1	196	196	0	0	262	285	372	374	240	240	211	217
2164A 29	M Hco3.N1.F1	L1	196	196	148	152	262	262	372	374	240	242	0	0
2164A 30	M Hco3.N1.F1	L1	196	196	0	0	262	285	372	372	240	240	211	211
2164A 31	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	374	240	242	211	211
2164A 32	M Hco3.N1.F1	L1	196	196	0	0	262	262	372	372	240	240	217	217
2164A 33	M Hco3.N1.F1	L1	196	196	0	0	262	285	372	372	240	240	211	211
2164A 34	M Hco3.N1.F1	L1	196	196	0	0	262	262	372	372	240	240	217	217
2164A 35	M Hco3.N1.F1	L1	196	196	0	0	262	262	372	372	240	242	217	217
2164A 40	M Hco3.N1.F1	L1	196	196	0	0	262	262	372	374	240	242	211	211
2164A 42	M Hco3.N1.F1	L1	196	196	148	148	262	285	372	372	240	240	211	217
2164A 43	M Hco3.N1.F1	L1	196	196	148	148	262	262	372	374	240	242	211	217
2164A 44	M Hco3.N1.F1	L1	196	196	148	152	262	262	372	374	240	242	211	217
2164A 45	M Hco3.N1.F1	L1	0	0	148	152	262	262	372	374	240	242	211	217
2164A 46	M Hco3.N1.F1	L1	196	196	148	148	262	262	372	374	240	242	211	211

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	N1 (♀parent)	Adult ♀	196	196	148	152	262	262	372	372	240	240	211	217
2274 B	M Hco3.N1.F2	L3	196	196	148	152	285	285	372	374	240	242	0	0
2274 J	M Hco3.N1.F2	L3	196	196	152	152	262	285	374	374	242	242	211	211
2274 S	M Hco3.N1.F2	L3	0	0	148	152	262	262	374	374	242	242	217	217
2274 u	M Hco3.N1.F2	L3	196	196	0	0	285	285	372	372	240	240	211	217
2274 v	M Hco3.N1.F2	L3	196	196	0	0	285	285	372	372	240	240	217	217
2758F1	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	262	372	374	240	240	217	217
2758F2	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	285	372	374	240	240	0	0
2758F3	M Hco3.N1.F2	Adult ♀	196	196	0	0	285	285	372	374	240	242	211	217
2758F4	M Hco3.N1.F2	Adult ♀	196	196	0	0	262	262	372	372	240	240	211	217
2758F5	M Hco3.N1.F2	Adult ♀	196	196	0	0	262	262	372	372	240	240	211	217
2758F6	M Hco3.N1.F2	Adult ♀	196	196	148	152	262	285	372	372	242	242	0	0
2758F7	M Hco3.N1.F2	Adult ♀	196	196	148	152	262	262	372	372	240	242	217	217
2758F8	M Hco3.N1.F2	Adult ♀	0	0	148	152	262	262	372	372	240	240	217	217
2758F9	M Hco3.N1.F2	Adult ♀	196	196	148	152	262	262	372	372	240	240	0	0
2758F11	M Hco3.N1.F2	Adult ♀	196	196	0	0	262	285	372	372	240	242	217	217
2758F12	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	285	372	374	240	240	211	217
2758F13	M Hco3.N1.F2	Adult ♀	196	196	0	0	262	285	372	372	240	240	211	217
2758F14	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	285	372	374	240	242	217	217
2758F15	M Hco3.N1.F2	Adult ♀	196	196	0	0	262	285	372	372	240	240	217	217
2758F16	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	285	372	372	240	240	217	217
2758F19	M Hco3.N1.F2	Adult ♀	196	196	0	0	262	262	372	372	240	240	217	217
2758F20	M Hco3.N1.F2	Adult ♀	196	196	0	0	262	285	372	374	240	240	211	217
2758F25	M Hco3.N1.F2	Adult ♀	0	0	152	152	262	285	372	374	240	242	217	217
2758M2	M Hco3.N1.F2	Adult ♂	196	196	0	0	262	262	372	372	240	240	211	217
2758M6	M Hco3.N1.F2	Adult ♂	0	0	148	152	262	285	372	372	240	240	211	217
2758M12	M Hco3.N1.F2	Adult ♂	0	0	148	152	285	285	372	372	240	242	217	217
2758M16	M Hco3.N1.F2	Adult ♂	0	0	148	148	262	262	372	374	242	242	211	217
2758M22	M Hco3.N1.F2	Adult ♂	0	0	148	148	262	262	372	372	240	242	211	217

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	N1 (♀parent)	Adult ♀	196	196	148	152	262	262	372	372	240	240	211	217
2758 E	M Hco3.N1.F3	L3	196	196	148	152	262	262	372	372	240	240	211	217
2758 F	M Hco3.N1.F3	L3	0	0	152	152	262	262	372	374	240	242	213	217
2758 M	M Hco3.N1.F3	L3	0	0	148	152	262	262	374	374	242	242	211	211
2758 w	M Hco3.N1.F3	L3	196	196	0	0	262	262	372	372	240	240	211	217
2758E.2	M Hco3.N1.F3	L3	196	196	0	0	262	262	372	372	240	240	211	217
2434 B	M Hco3.N1.F4	L3	196	196	148	152	262	262	372	374	240	242	211	211
2434 D	M Hco3.N1.F4	L3	196	196	148	152	262	262	372	374	240	242	211	217
2434 E	M Hco3.N1.F4	L3	196	196	152	152	262	262	372	372	240	240	217	217
2434 F	M Hco3.N1.F4	L3	196	196	152	152	262	262	372	374	240	242	217	217
2434 J	M Hco3.N1.F4	L3	196	196	148	152	262	262	372	374	240	242	217	217
2434 K	M Hco3.N1.F4	L3	196	196	152	152	262	285	372	374	240	242	217	217
2434 L	M Hco3.N1.F4	L3	196	196	148	152	262	262	374	374	242	242	211	217

Appendix 4.4ii): Microsatellite genotypes of the founding N2 female parent and individual MHco3.N2 filial nematodes. Data are shown for individual worms where one or less of six microsatellites were not amplified.

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2144G Fem	N2 (♀parent)	Adult ♀	232	232	148	148	262	288	372	374	242	246	211	217
2144G 1	M Hco3.N2.F1	L1	232	232	148	148	262	285	374	374	240	242	209	211
2144G 2	M Hco3.N2.F1	L1	0	0	148	148	288	288	372	374	240	240	211	215
2144G 12	M Hco3.N2.F1	L1	232	232	148	148	262	285	372	374	242	246	209	211
2144G 13	M Hco3.N2.F1	L1	232	232	148	148	262	288	372	374	242	246	209	217
2144G 15	M Hco3.N2.F1	L1	232	232	148	148	288	288	372	374	240	246	217	217
2144G 16	M Hco3.N2.F1	L1	232	232	148	148	262	285	374	374	242	242	217	217
2144G 23	M Hco3.N2.F1	L1	232	232	148	148	288	288	372	374	240	246	0	0
2220F1	M Hco3.N2.F1	Adult ♀	232	232	148	148	262	285	372	374	240	246	209	217
2220F2	M Hco3.N2.F1	Adult ♀	232	232	148	148	288	288	372	374	240	246	215	217
2220F3	M Hco3.N2.F1	Adult ♀	232	232	148	148	262	285	372	374	240	246	215	217
2220F4	M Hco3.N2.F1	Adult ♀	232	232	0	0	262	285	374	374	240	242	211	211
2220F5	M Hco3.N2.F1	Adult ♀	232	232	148	148	288	288	374	374	240	242	215	217
2220M1	M Hco3.N2.F1	Adult ♂	232	232	0	0	288	288	374	374	242	242	209	211
2220 J	M Hco3.N2.F2	L3	196	196	148	148	288	288	374	374	242	242	0	0
2220 K	M Hco3.N2.F2	L3	196	196	148	148	285	285	372	374	240	246	0	0
2220 b	M Hco3.N2.F2	L3	232	232	0	0	285	288	372	372	246	246	215	215
2220 d	M Hco3.N2.F2	L3	232	232	0	0	288	288	372	372	246	246	209	217
2220Q2	M Hco3.N2.F2	L3	240	240	148	148	285	285	374	374	242	246	0	0
2427 D	M Hco3.N2.F3	L3	232	232	148	148	285	285	374	374	242	242	215	215
2427 L	M Hco3.N2.F3	L3	232	232	148	148	285	285	372	374	240	240	211	211
2427 M	M Hco3.N2.F3	L3	0	0	148	148	288	288	374	374	240	242	217	217
2427 t	M Hco3.N2.F3	L3	232	232	0	0	285	285	372	372	246	246	215	215

Appendix 4.4iii): Microsatellite genotypes of the founding N1 and N2 female parents and individual MHco3 nematodes. Data are shown for individual worms where one or less of six microsatellites were not amplified.

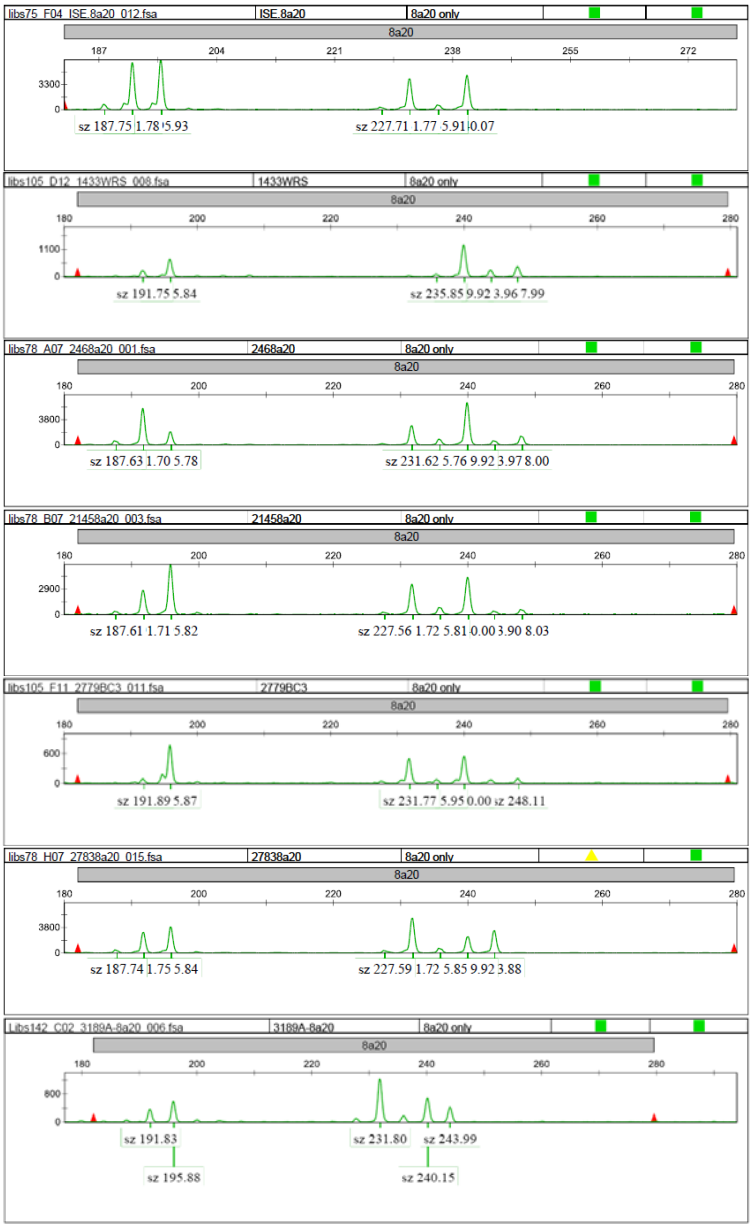
ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	**N1 (♀parent)**	**Adult ♀**	**196**	**196**	**148**	**152**	**262**	**262**	**372**	**372**	**240**	**240**	**211**	**217**
2144G Fem	**N2 (♀parent)**	**Adult ♀**	**232**	**232**	**148**	**148**	**262**	**288**	**372**	**374**	**242**	**246**	**211**	**217**
GL1659	MHco3	L3	192	232	148	152	262	262	372	374	242	246	209	211
GL1660	MHco3	L3	232	240	148	148	285	285	372	374	240	242	211	211
GL1661	MHco3	L3	232	240	148	152	262	285	372	374	240	242	211	213
GL1662	MHco3	L3	192	232	148	148	262	262	372	372	240	240	213	213
GL1663	MHco3	L3	192	196	152	152	262	262	372	374	240	242	211	211
GL1664	MHco3	L3	192	232	148	152	285	285	372	374	240	242	213	213
GL1666	MHco3	L3	196	196	148	152	285	285	372	374	240	242	211	217
GL1667	MHco3	L3	192	232	148	152	262	285	372	372	240	240	211	213
GL1668	MHco3	L3	192	232	148	152	285	288	372	372	240	240	211	211
GL1669	MHco3	L3	192	240	148	152	262	288	372	374	240	240	209	213
GL1670	MHco3	L3	192	192	148	148	285	285	372	374	240	242	213	215
GL1671	MHco3	L3	196	196	148	152	262	285	374	374	242	242	217	217
GL1672	MHco3	L3	192	232	148	152	262	285	374	374	242	242	213	213
GL1673	MHco3	L3	240	240	148	152	262	288	372	374	240	242	213	217
GL1676	MHco3	L3	192	240	148	152	262	285	372	372	240	240	215	215
GL1677	MHco3	L3	196	196	148	148	262	285	372	372	240	246	213	213
GL1678	MHco3	L3	192	240	152	152	262	285	372	372	240	240	213	213
GL1679	MHco3	L3	192	232	148	152	262	262	372	372	240	240	213	213
GL1680	MHco3	L3	192	240	148	148	262	262	372	372	240	240	213	213
GL1681	MHco3	L3	192	232	148	152	262	262	372	374	240	242	211	213
GL1682	MHco3	L3	232	232	148	148	262	285	372	372	240	240	217	217
GL1683	MHco3	L3	192	232	148	148	262	285	372	374	240	242	211	213
GL1684	MHco3	L3	232	232	148	152	262	285	372	372	240	240	0	0
GL1685	MHco3	L3	232	240	148	148	262	285	372	374	240	242	211	211
GL1686	MHco3	L3	232	232	148	152	262	285	372	372	240	240	211	217
GL1687	MHco3	L3	232	240	148	148	262	285	372	374	240	242	213	213
GL1688	MHco3	L3	240	240	148	152	262	285	374	374	242	242	209	213
GL1689	MHco3	L3	232	240	148	152	262	285	374	374	242	242	213	217
GL1690	MHco3	L3	192	196	148	152	262	285	372	374	240	240	211	217

Appendix 4.5: Microsatellite genotypes of the founding N1 and N2 female parents, individual MHco3.N1 and MHco3.N2 filial nematodes and individual MHco3 (ISE) nematodes. Data are shown for individual worms where six of six microsatellites were amplified.

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	N1 (♀parent)	Adult ♀	196	196	148	152	262	262	372	372	240	240	211	217
2164A 2	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	374	240	242	211	217
2164A 5	M Hco3.N1.F1	L1	196	196	148	152	262	262	372	372	240	240	211	217
2164A 6	M Hco3.N1.F1	L1	196	196	148	148	262	285	372	372	240	240	211	217
2164A 7	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	372	240	240	211	217
2164A 8	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	372	240	240	217	217
2164A 9	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	372	240	240	211	217
2164A 17	M Hco3.N1.F1	L1	196	196	148	148	262	262	372	372	240	240	211	217
2164A 23	M Hco3.N1.F1	L1	196	196	148	152	262	262	372	372	240	240	211	211
2164A 31	M Hco3.N1.F1	L1	196	196	148	152	262	285	372	374	240	242	211	211
2164A 42	M Hco3.N1.F1	L1	196	196	148	148	262	285	372	372	240	240	211	217
2164A 43	M Hco3.N1.F1	L1	196	196	148	148	262	262	372	374	240	242	211	217
2164A 44	M Hco3.N1.F1	L1	196	196	148	152	262	262	372	374	240	242	211	217
2164A 46	M Hco3.N1.F1	L1	196	196	148	148	262	262	372	374	240	242	211	211
2274 J	M Hco3.N1.F2	L3	196	196	152	152	262	285	374	374	242	242	211	211
2758F1	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	262	372	374	240	240	217	217
2758F7	M Hco3.N1.F2	Adult ♀	196	196	148	152	262	262	372	372	240	242	217	217
2758F12	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	285	372	374	240	240	211	217
2758F14	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	285	372	374	240	242	217	217
2758F16	M Hco3.N1.F2	Adult ♀	196	196	152	152	262	285	372	372	240	240	217	217
2758 E	M Hco3.N1.F3	L3	196	196	148	152	262	262	372	372	240	240	211	217
2434 B	M Hco3.N1.F4	L3	196	196	148	152	262	262	372	374	240	242	211	211
2434 D	M Hco3.N1.F4	L3	196	196	148	152	262	262	372	374	240	242	211	217
2434 E	M Hco3.N1.F4	L3	196	196	152	152	262	262	372	372	240	240	217	217
2434 F	M Hco3.N1.F4	L3	196	196	152	152	262	262	372	374	240	242	217	217
2434 J	M Hco3.N1.F4	L3	196	196	148	152	262	262	372	374	240	242	217	217
2434 K	M Hco3.N1.F4	L3	196	196	152	152	262	285	372	374	240	242	217	217
2434 L	M Hco3.N1.F4	L3	196	196	148	152	262	262	374	374	242	242	211	217

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2144G Fem	N2 (♀parent)	Adult ♀	232	232	148	148	262	288	372	374	242	246	211	217
2144G 1	M Hco3.N2.F1	L1	232	232	148	148	262	285	374	374	240	242	209	211
2144G 12	M Hco3.N2.F1	L1	232	232	148	148	262	285	372	374	242	246	209	211
2144G 13	M Hco3.N2.F1	L1	232	232	148	148	262	288	372	374	242	246	209	217
2144G 15	M Hco3.N2.F1	L1	232	232	148	148	288	288	372	374	240	246	217	217
2144G 16	M Hco3.N2.F1	L1	232	232	148	148	262	285	374	374	242	242	217	217
2220F1	M Hco3.N2.F1	Adult ♀	232	232	148	148	262	285	372	374	240	246	209	217
2220F2	M Hco3.N2.F1	Adult ♀	232	232	148	148	288	288	372	374	240	246	215	217
2220F3	M Hco3.N2.F1	Adult ♀	232	232	148	148	262	285	372	374	240	246	215	217
2220F5	M Hco3.N2.F1	Adult ♀	232	232	148	148	288	288	374	374	240	242	215	217
2427 D	M Hco3.N2.F3	L3	232	232	148	148	285	285	374	374	242	242	215	215
2427 L	M Hco3.N2.F3	L3	232	232	148	148	285	285	372	374	240	240	211	211

ID	Line	Stage	8a20		Hcms36		3561		X 182762		X 240993		Hcms25	
2164A Fem	N1 (♀parent)	Adult ♀	196	196	148	152	262	262	372	372	240	240	211	217
2144G Fem	N2 (♀parent)	Adult ♀	232	232	148	148	262	288	372	374	242	246	211	217
GL1659	MHco3	L3	192	232	148	152	262	262	372	374	242	246	209	211
GL1660	MHco3	L3	232	240	148	148	285	285	372	374	240	242	211	211
GL1661	MHco3	L3	232	240	148	152	262	285	372	374	240	242	211	213
GL1662	MHco3	L3	192	232	148	148	262	262	372	372	240	240	213	213
GL1663	MHco3	L3	192	196	152	152	262	262	372	374	240	242	211	211
GL1664	MHco3	L3	192	232	148	152	285	285	372	374	240	242	213	213
GL1666	MHco3	L3	196	196	148	152	285	285	372	374	240	242	211	217
GL1667	MHco3	L3	192	232	148	152	262	285	372	372	240	240	211	213
GL1668	MHco3	L3	192	232	148	152	285	288	372	372	240	240	211	211
GL1669	MHco3	L3	192	240	148	152	262	288	372	374	240	240	209	213
GL1670	MHco3	L3	192	192	148	148	285	285	372	374	240	242	213	215
GL1671	MHco3	L3	196	196	148	152	262	285	374	374	242	242	217	217
GL1672	MHco3	L3	192	232	148	152	262	285	374	374	242	242	213	213
GL1673	MHco3	L3	240	240	148	152	262	288	372	374	240	242	213	217
GL1676	MHco3	L3	192	240	148	152	262	285	372	372	240	240	215	215
GL1677	MHco3	L3	196	196	148	148	262	285	372	372	240	246	213	213
GL1678	MHco3	L3	192	240	152	152	262	285	372	372	240	240	213	213
GL1679	MHco3	L3	192	232	148	152	262	262	372	372	240	240	213	213
GL1680	MHco3	L3	192	240	148	148	262	262	372	372	240	240	213	213
GL1681	MHco3	L3	192	232	148	152	262	262	372	374	240	242	211	213
GL1682	MHco3	L3	232	232	148	148	262	285	372	372	240	240	217	217
GL1683	MHco3	L3	192	232	148	148	262	285	372	374	240	242	211	213
GL1685	MHco3	L3	232	240	148	148	262	285	372	374	240	242	211	211
GL1686	MHco3	L3	232	232	148	152	262	285	372	372	240	240	211	217
GL1687	MHco3	L3	232	240	148	148	262	285	372	374	240	242	213	213
GL1688	MHco3	L3	240	240	148	152	262	285	374	374	242	242	209	213
GL1689	MHco3	L3	232	240	148	152	262	285	374	374	242	242	213	217
GL1690	MHco3	L3	192	196	148	152	262	285	372	374	240	240	211	217



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

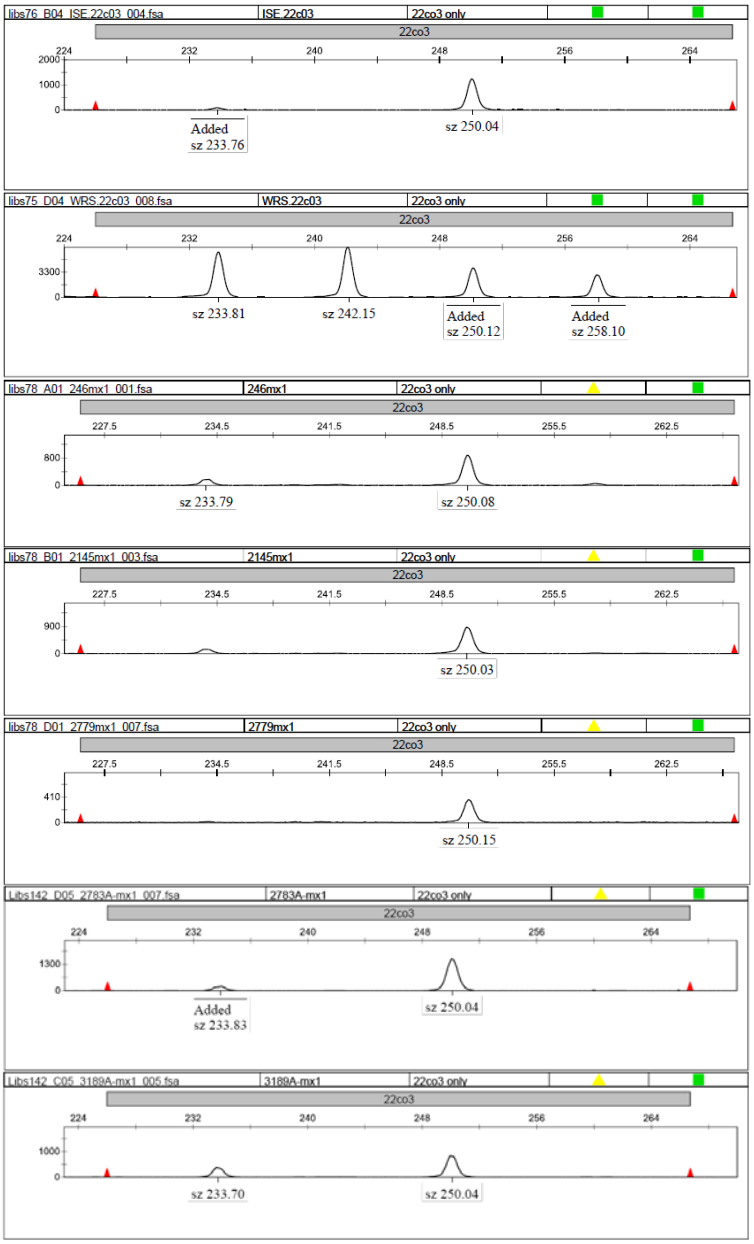
MHco3/4.BC$_1$

MHco3/4.BC$_2$

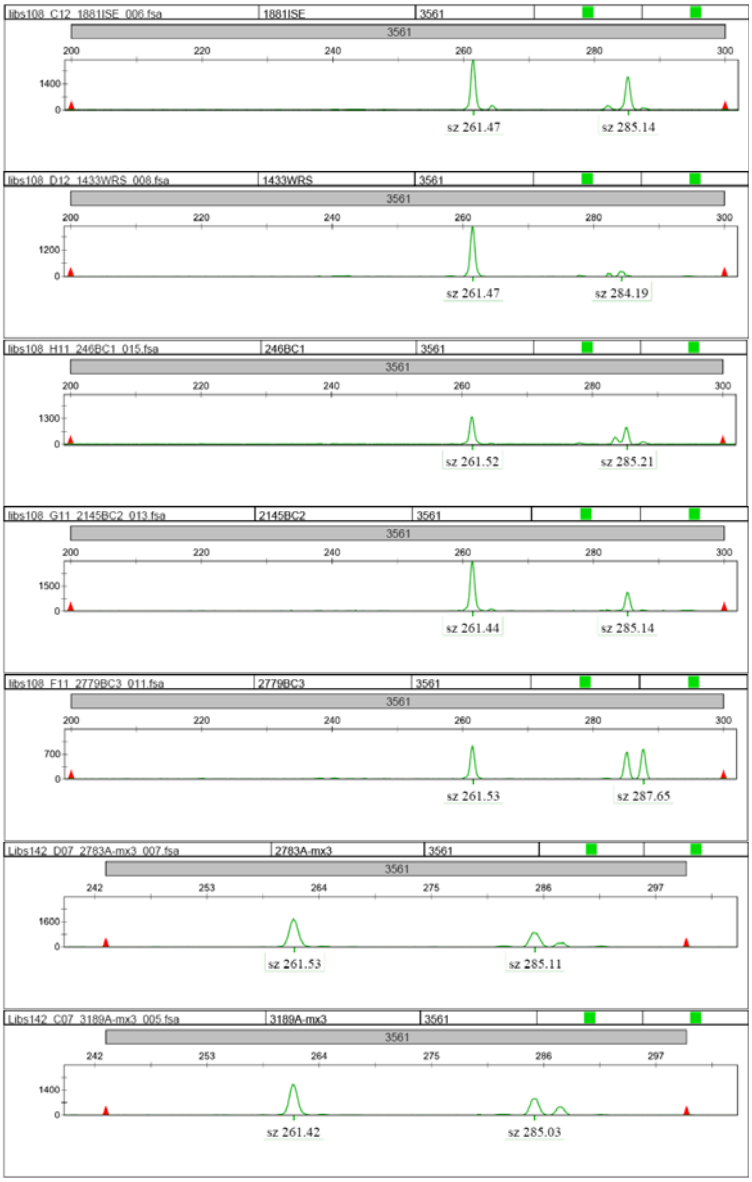
MHco3/4.BC$_3$

MHco3/4.BC$_4$

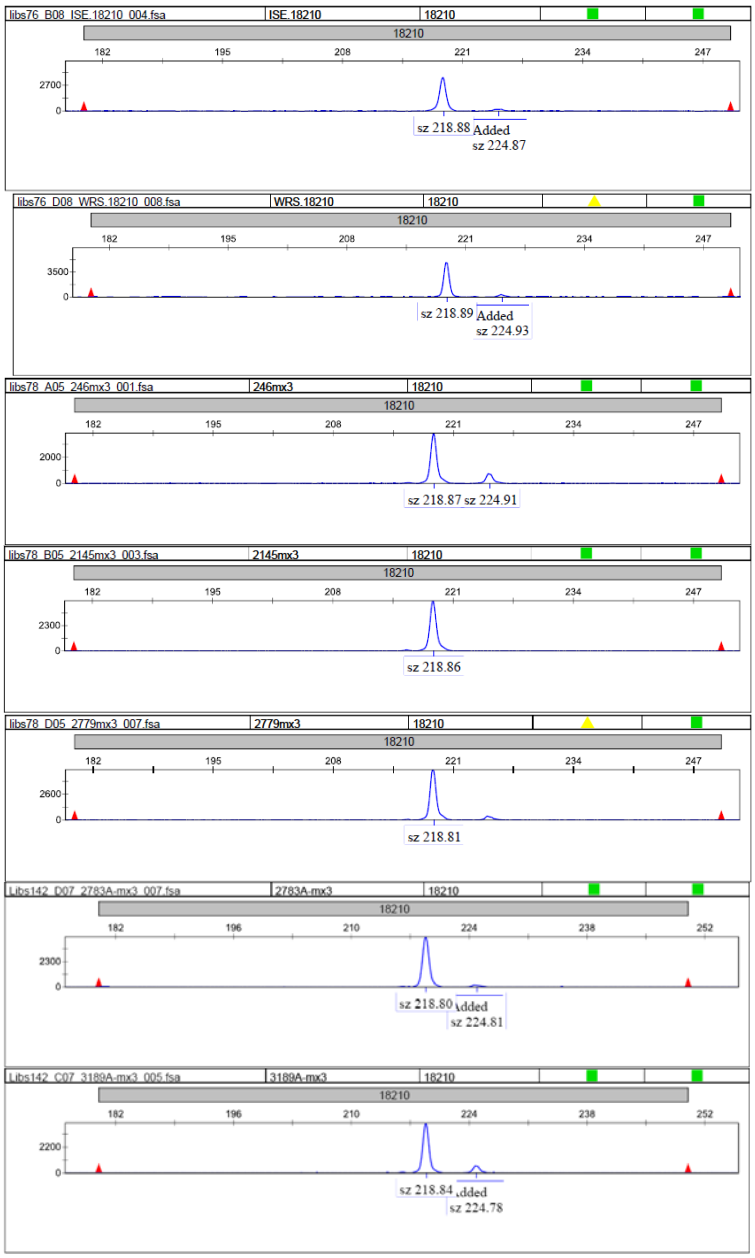
Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **Hcms8a20** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



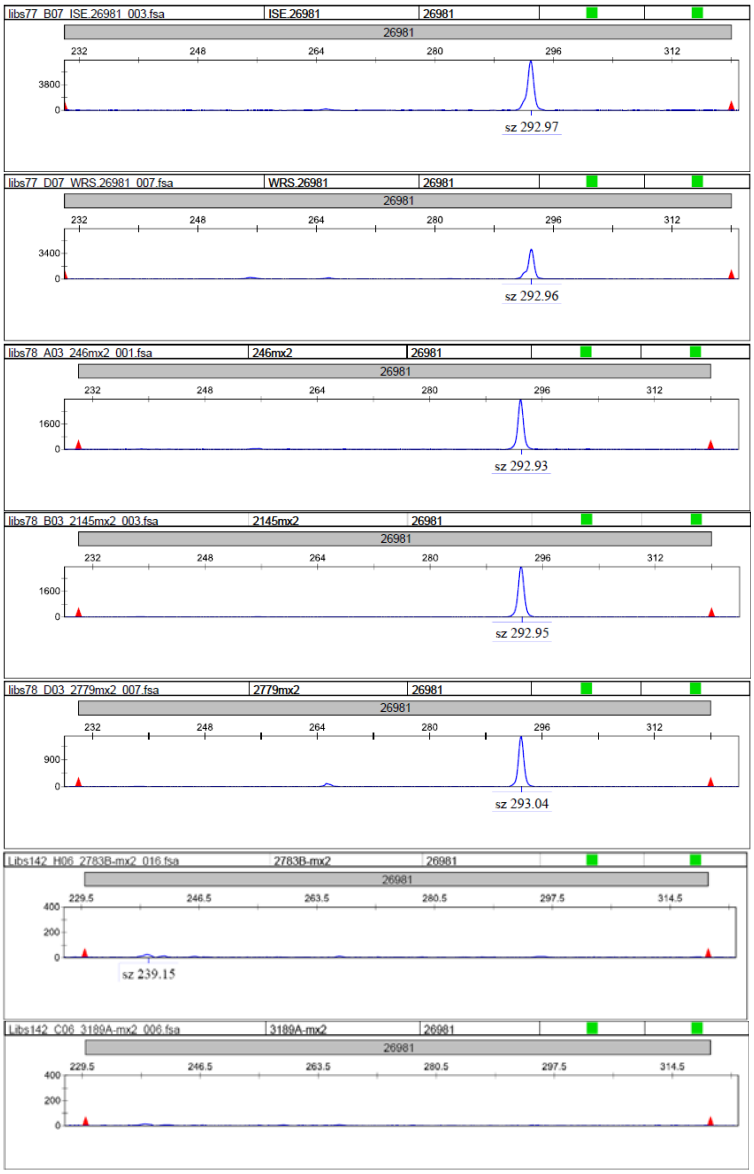
Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **Hcms22c03** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



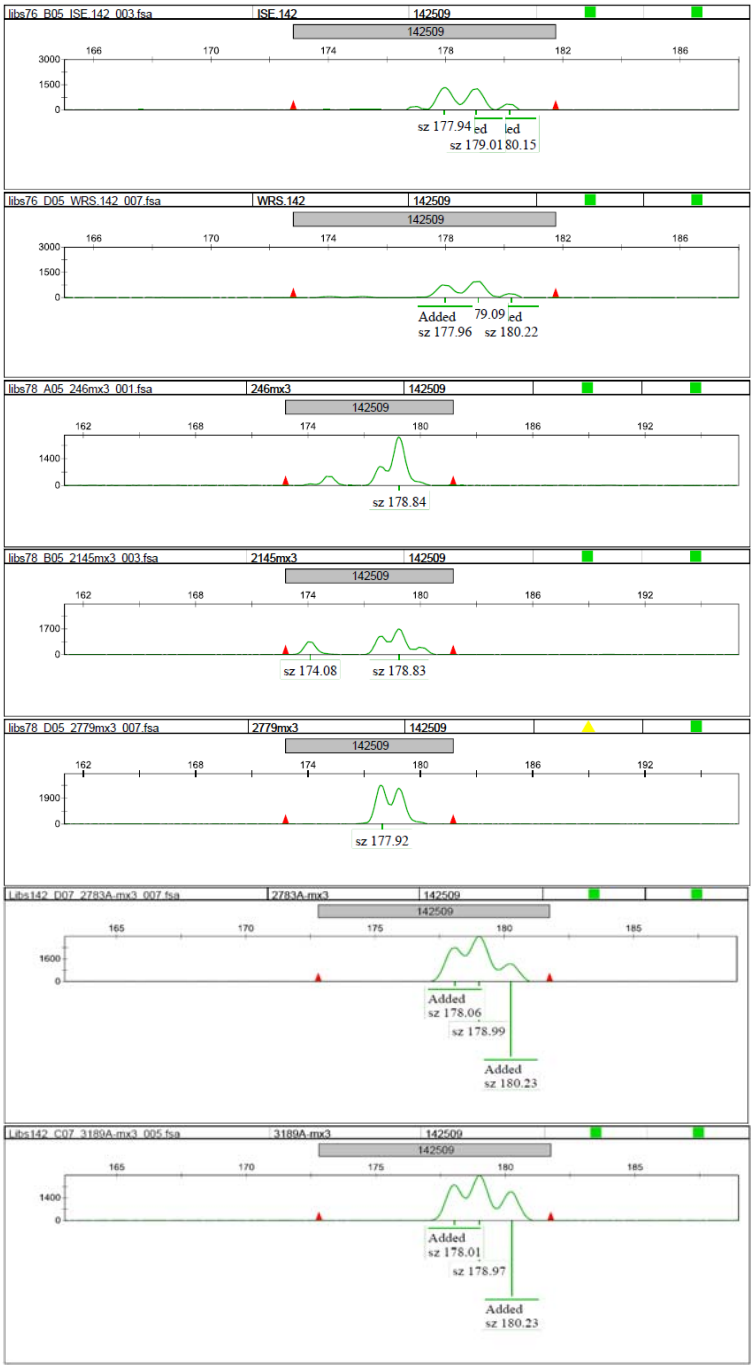
Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **3561** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



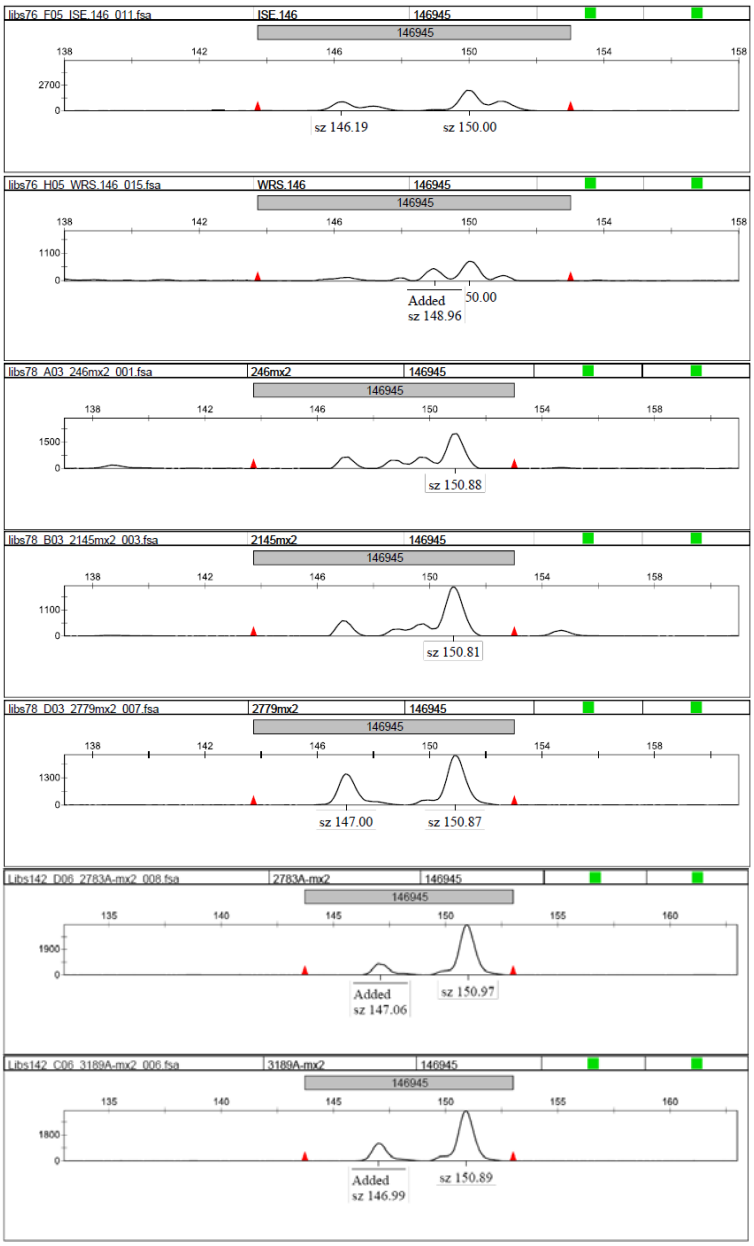
Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **18210** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



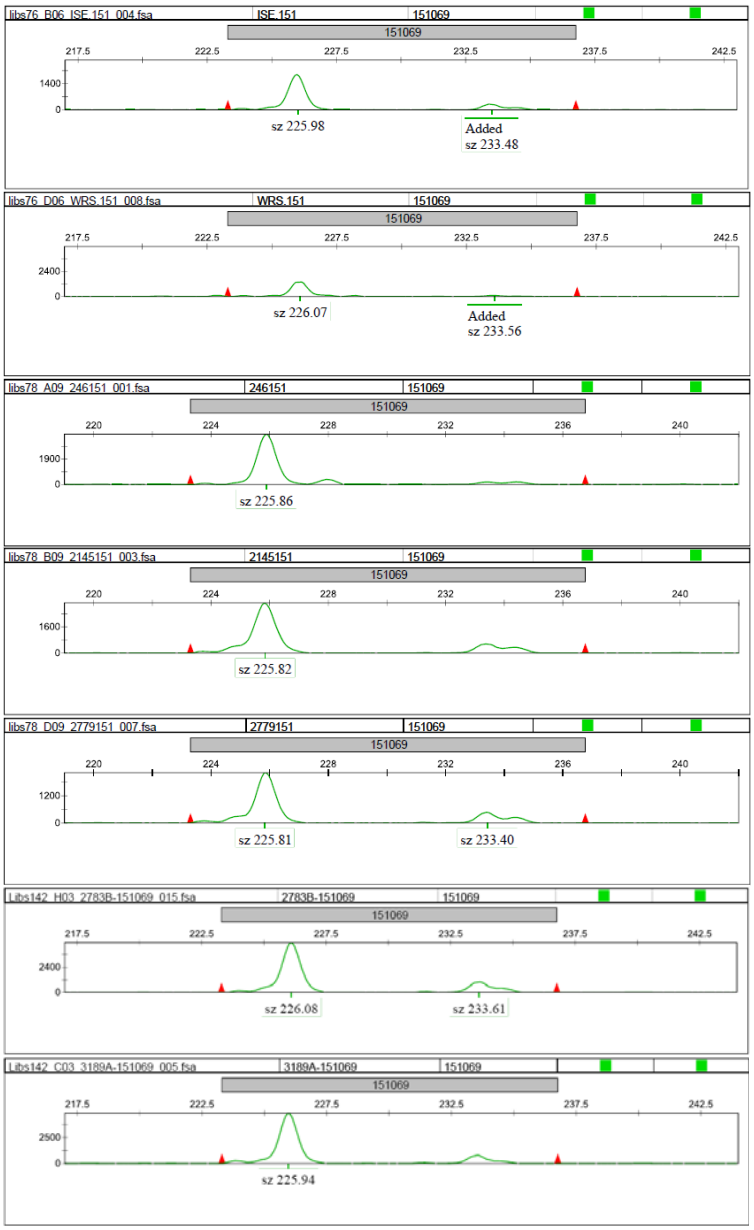
Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **26981** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



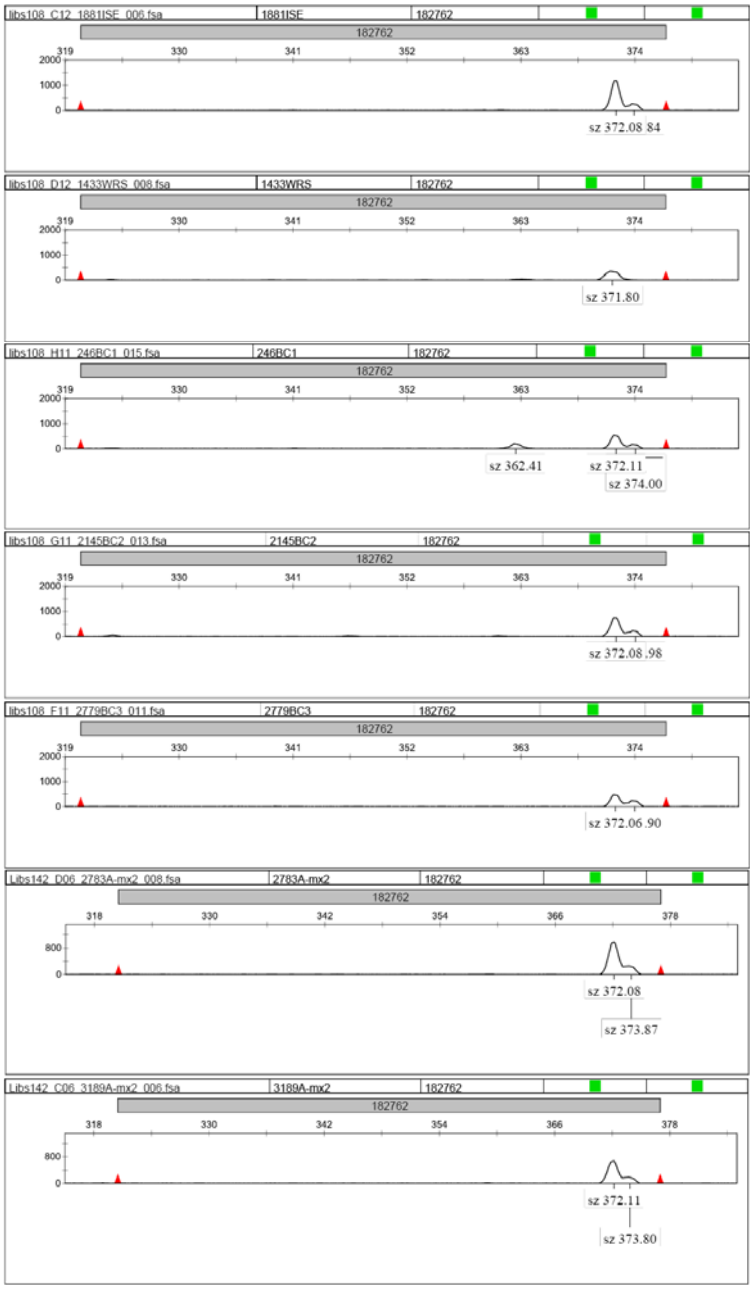
Appendix 5.1: Microsatellite Genescan traces for microsatellite marker X**142** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.1: Microsatellite Genescan traces for microsatellite marker X**146** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **X151** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

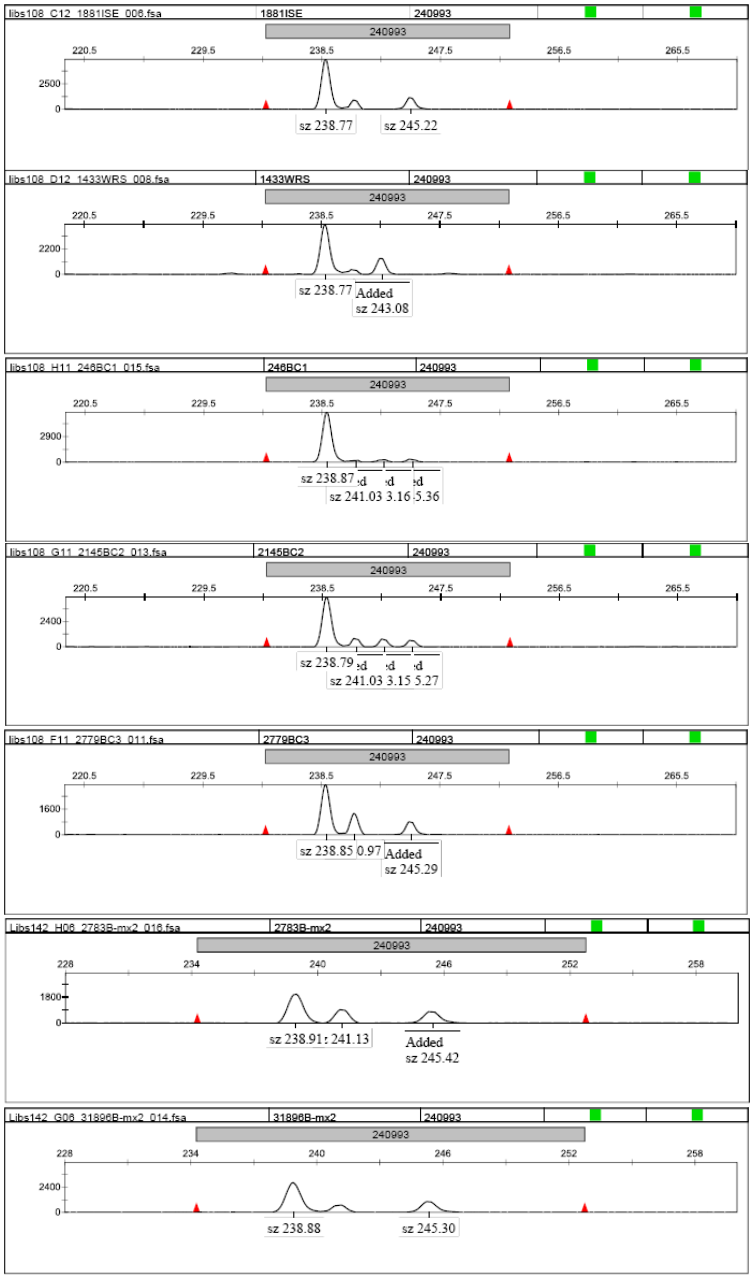
MHco3/4.BC$_1$

MHco3/4.BC$_2$

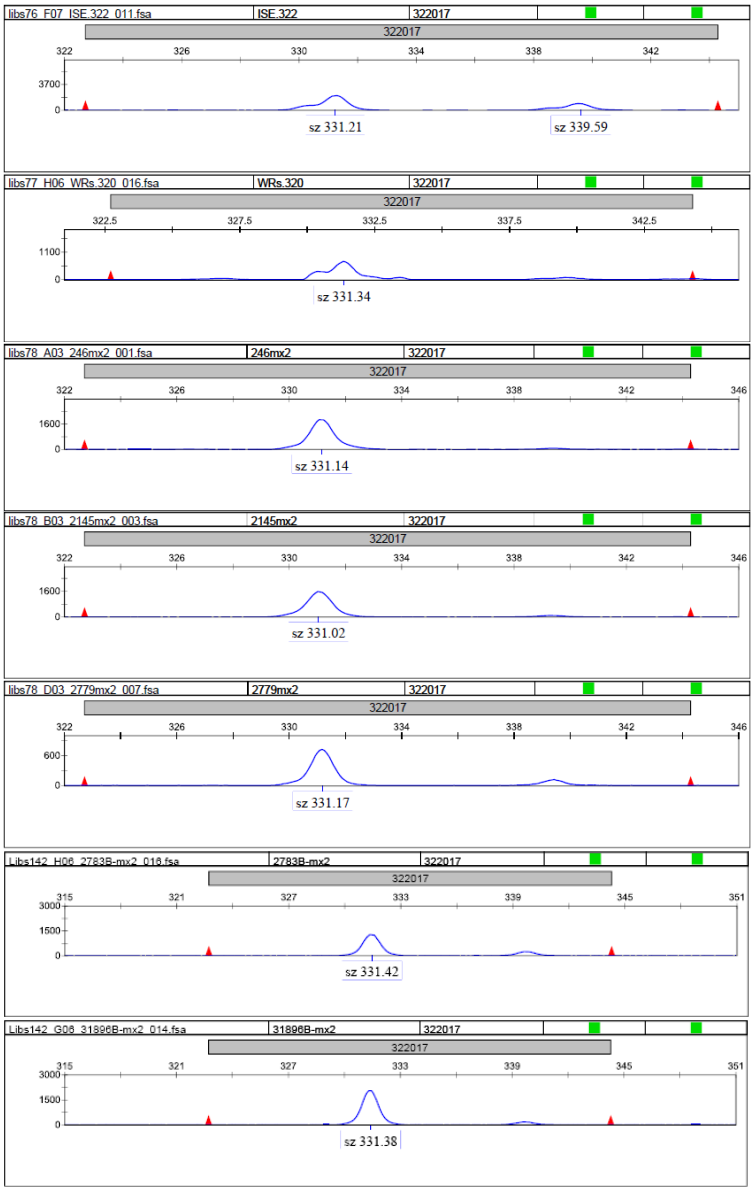
MHco3/4.BC$_3$

MHco3/4.BC$_4$

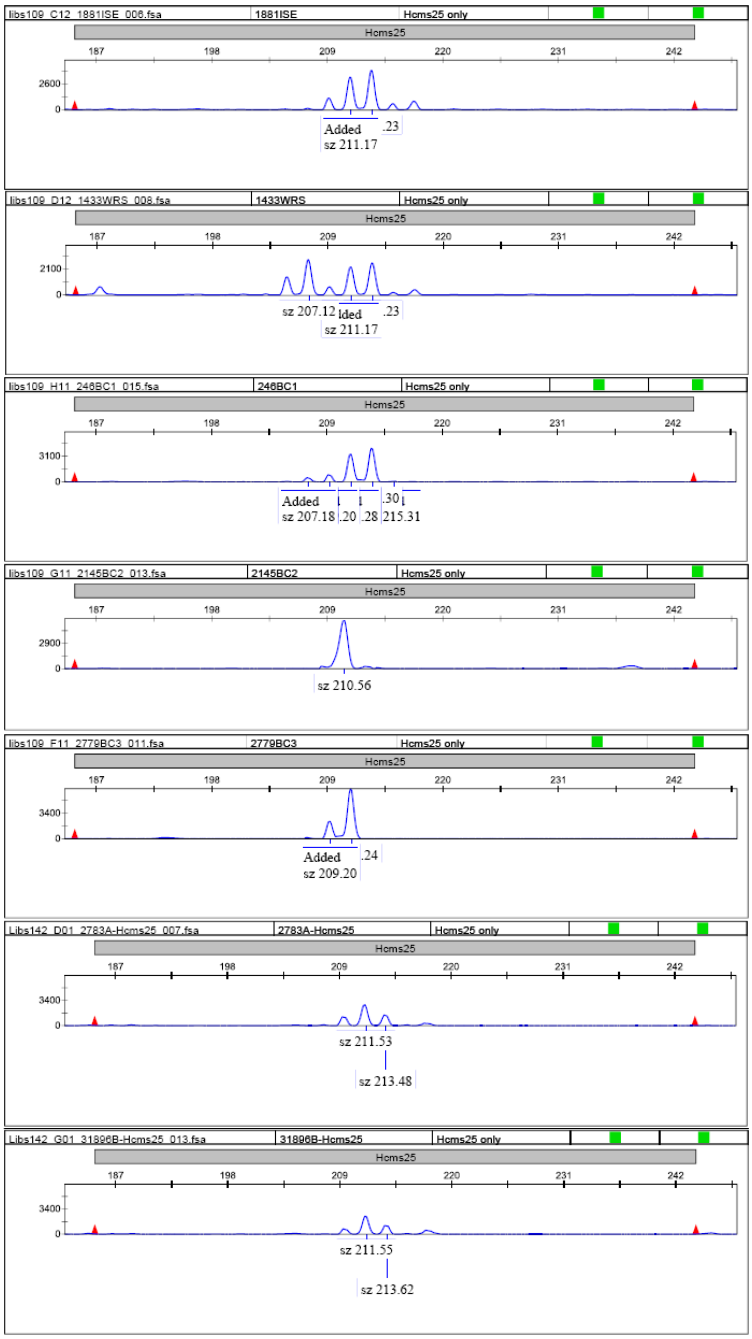
Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **X182** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **X256** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **X337** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

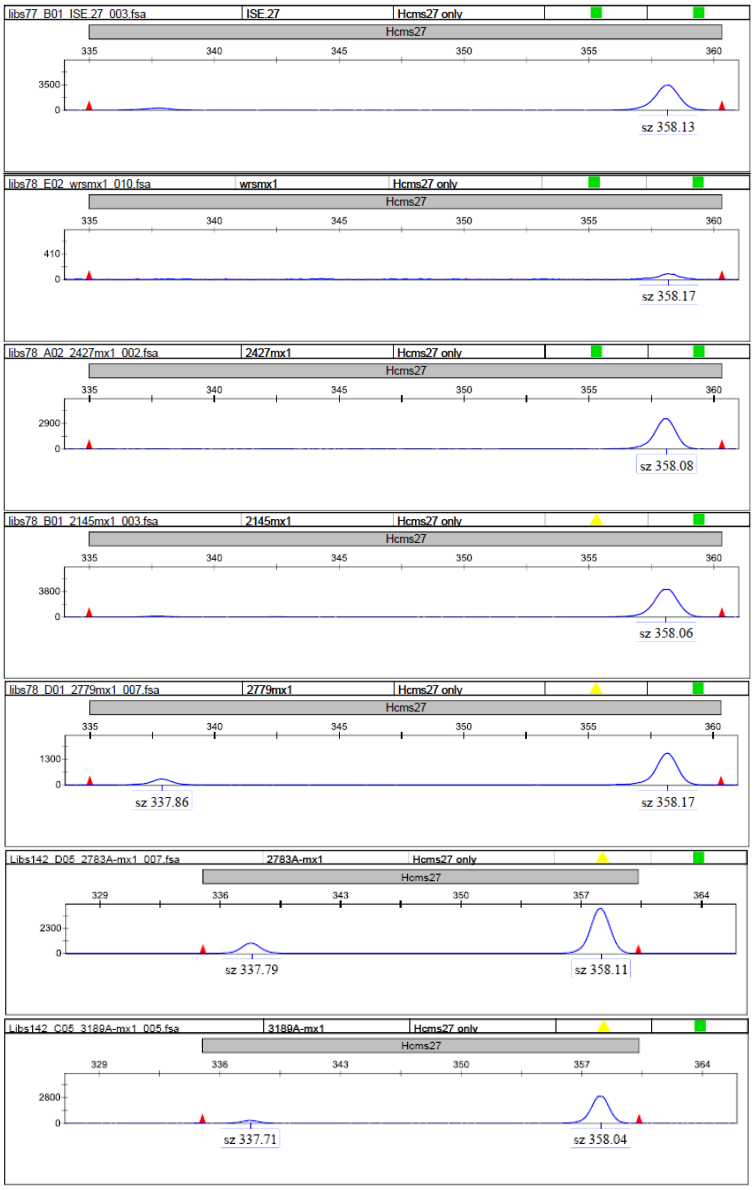
MHco3/4.BC$_1$

MHco3/4.BC$_2$

MHco3/4.BC$_3$

MHco3/4.BC$_4$

Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **Hcms25** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

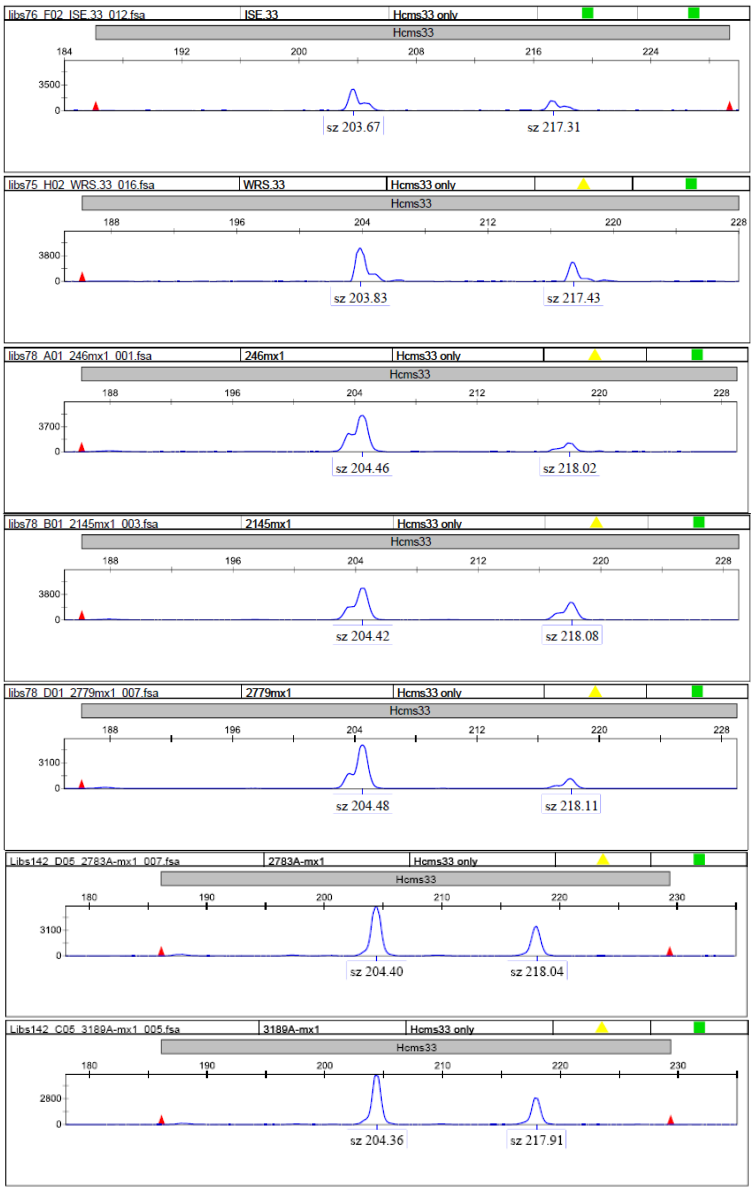
MHco3/4.BC_1

MHco3/4.BC_2

MHco3/4.BC_3

MHco3/4.BC_4

Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **Hcms27** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

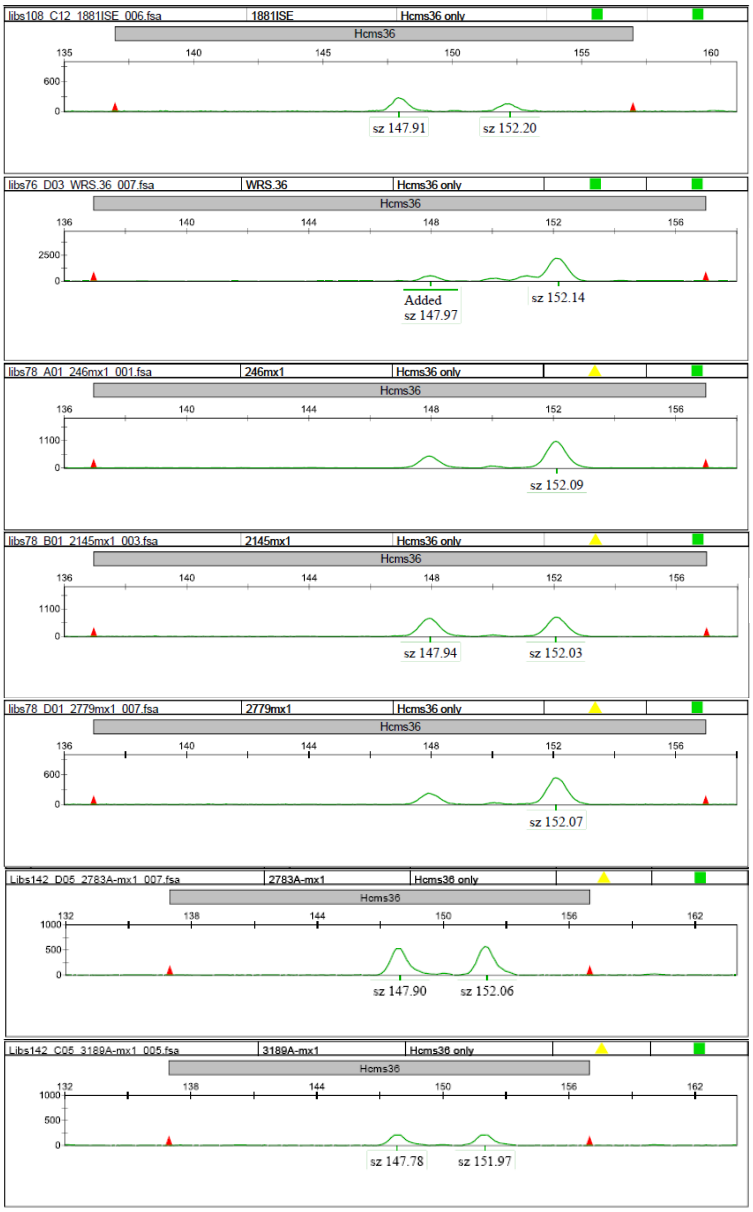
MHco3/4.BC$_1$

MHco3/4.BC$_2$

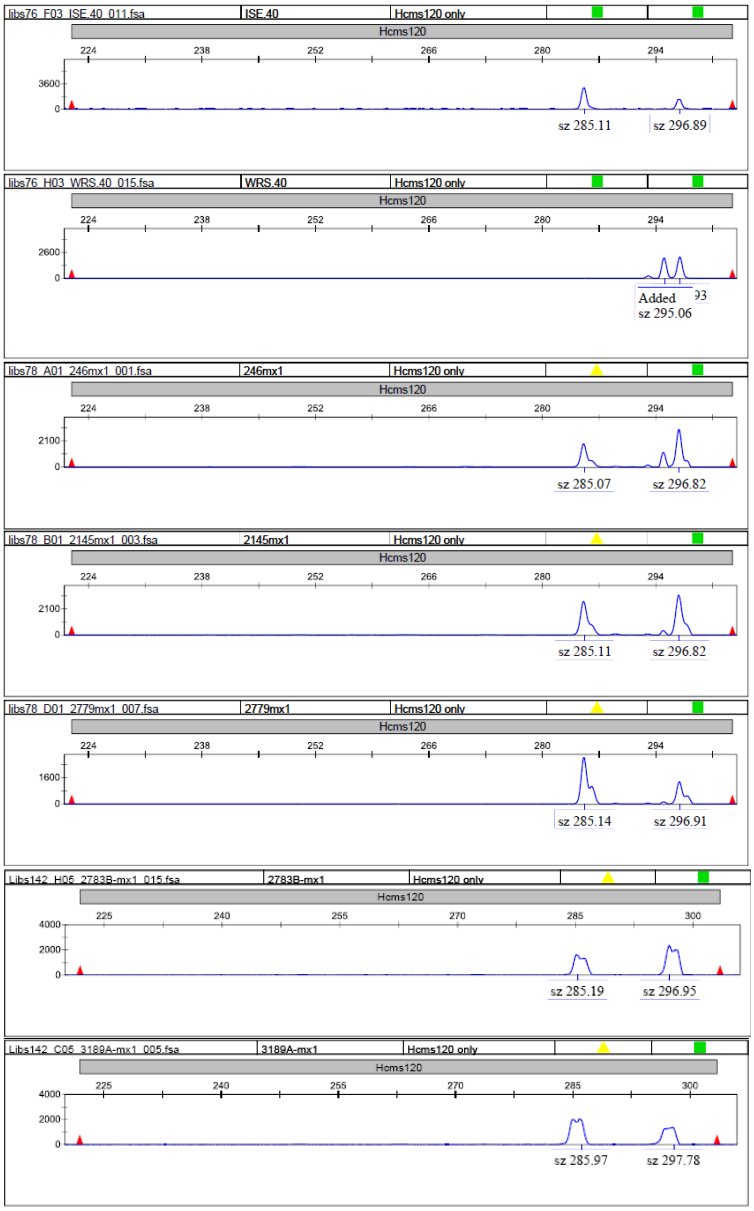
MHco3/4.BC$_3$

MHco3/4.BC$_4$

Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **Hcms33** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **Hcms36** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

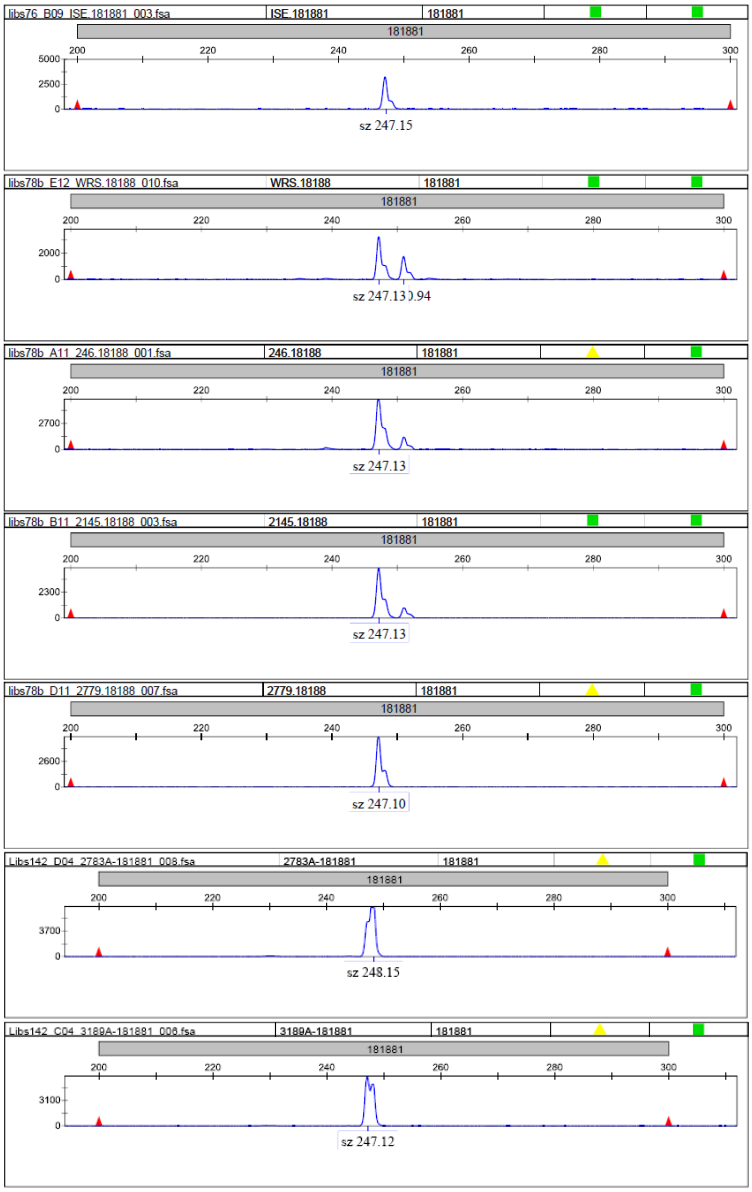
MHco3/4.BC$_1$

MHco3/4.BC$_2$

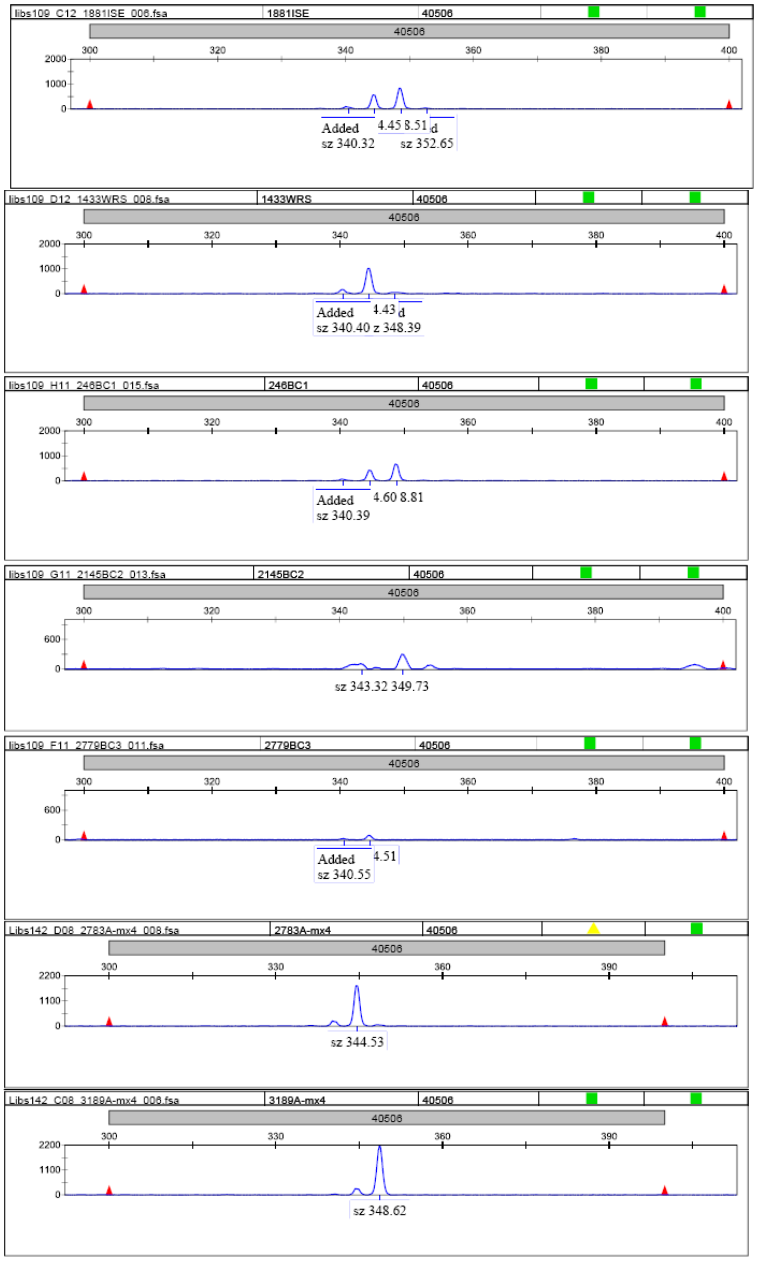
MHco3/4.BC$_3$

MHco3/4.BC$_4$

Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **Hcms40** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **181881** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco4 (WRS)

MHco3/4

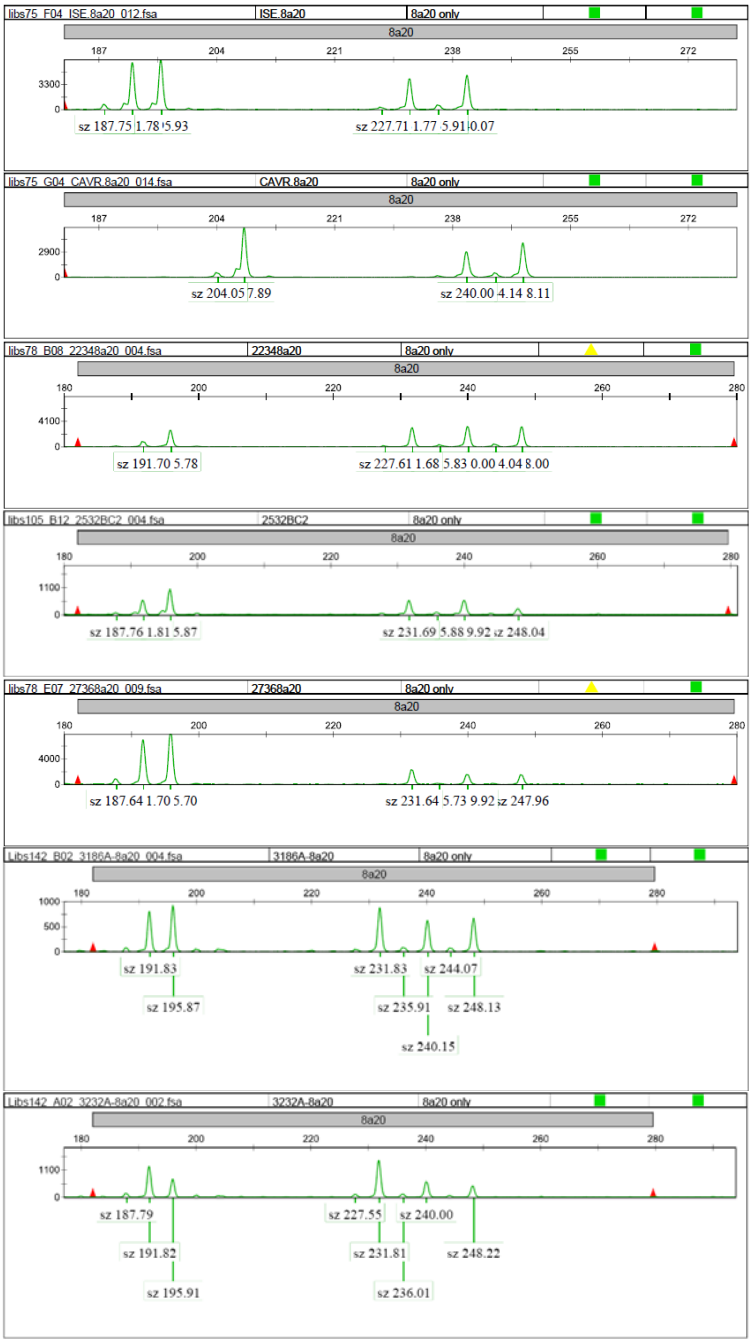
MHco3/4.BC$_1$

MHco3/4.BC$_2$

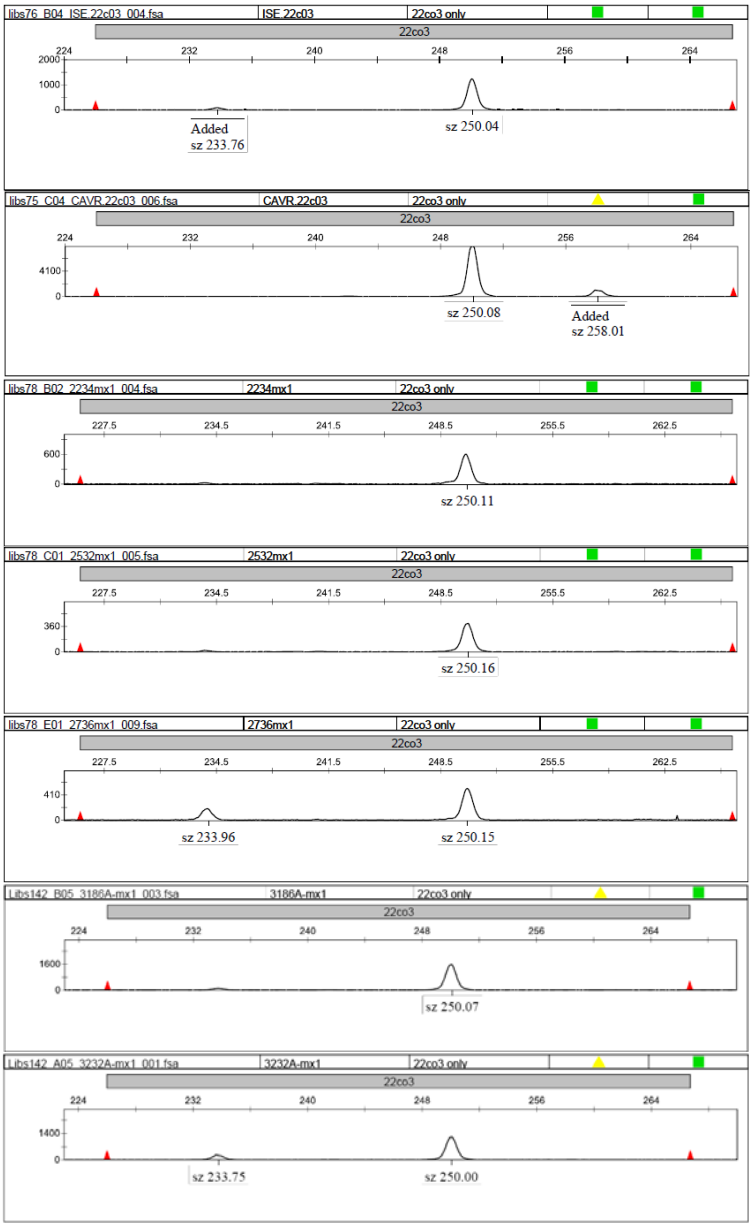
MHco3/4.BC$_3$

MHco3/4.BC$_4$

Appendix 5.1: Microsatellite Genescan traces for microsatellite marker **40506** for bulk lysates of the parental MHco3 (ISE) and MHco4 (WRS) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **Hcms8a20** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco10 (CAVR)

MHco3/10

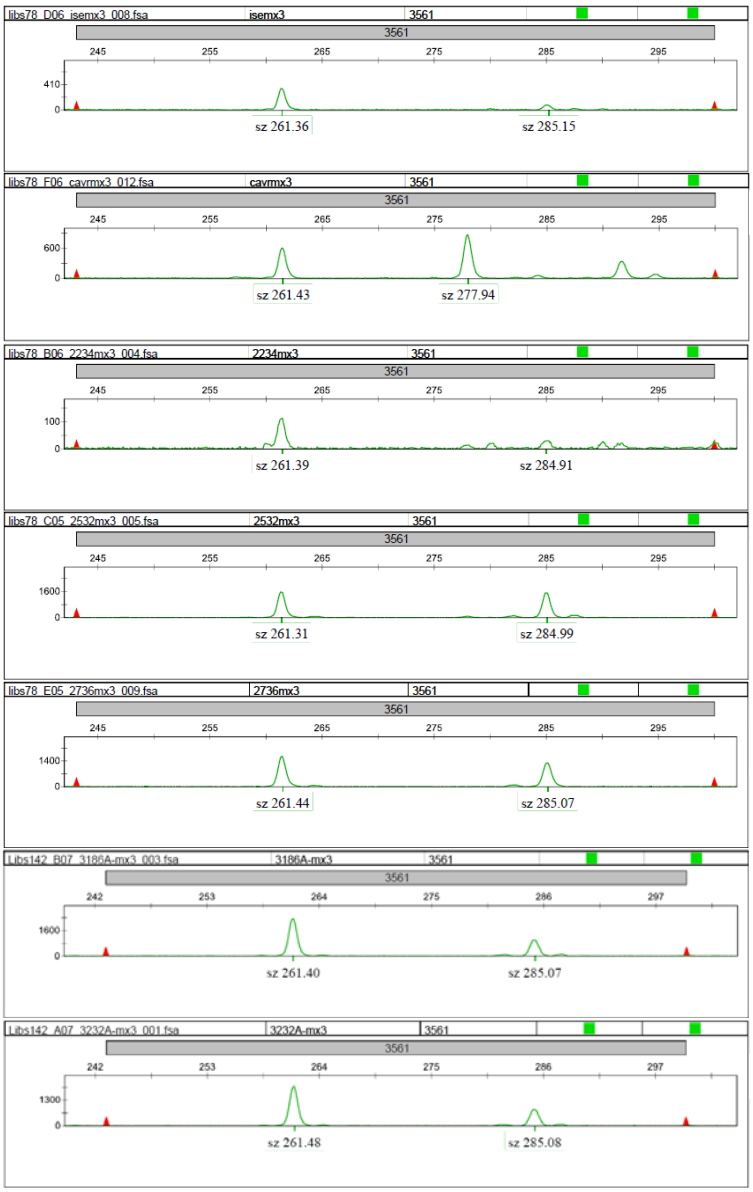
MHco3/10.BC$_1$

MHco3/10.BC$_2$

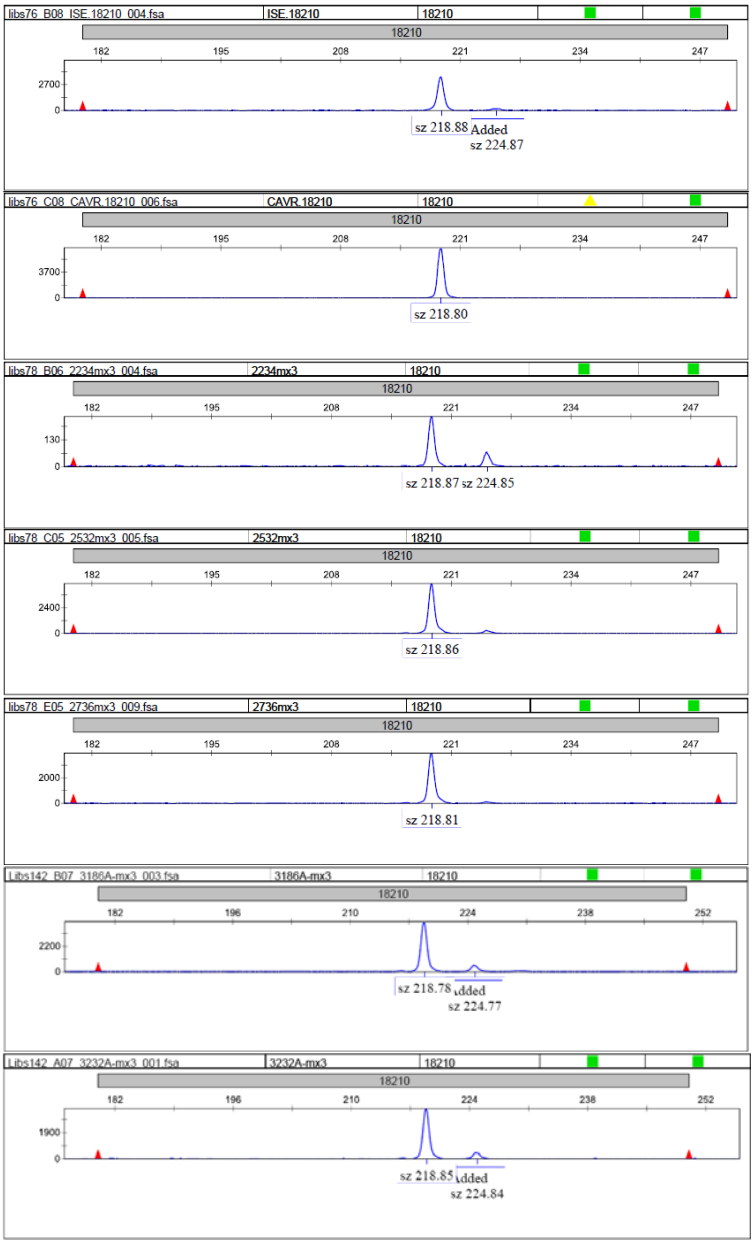
MHco3/10.BC$_3$

MHco3/10.BC$_4$

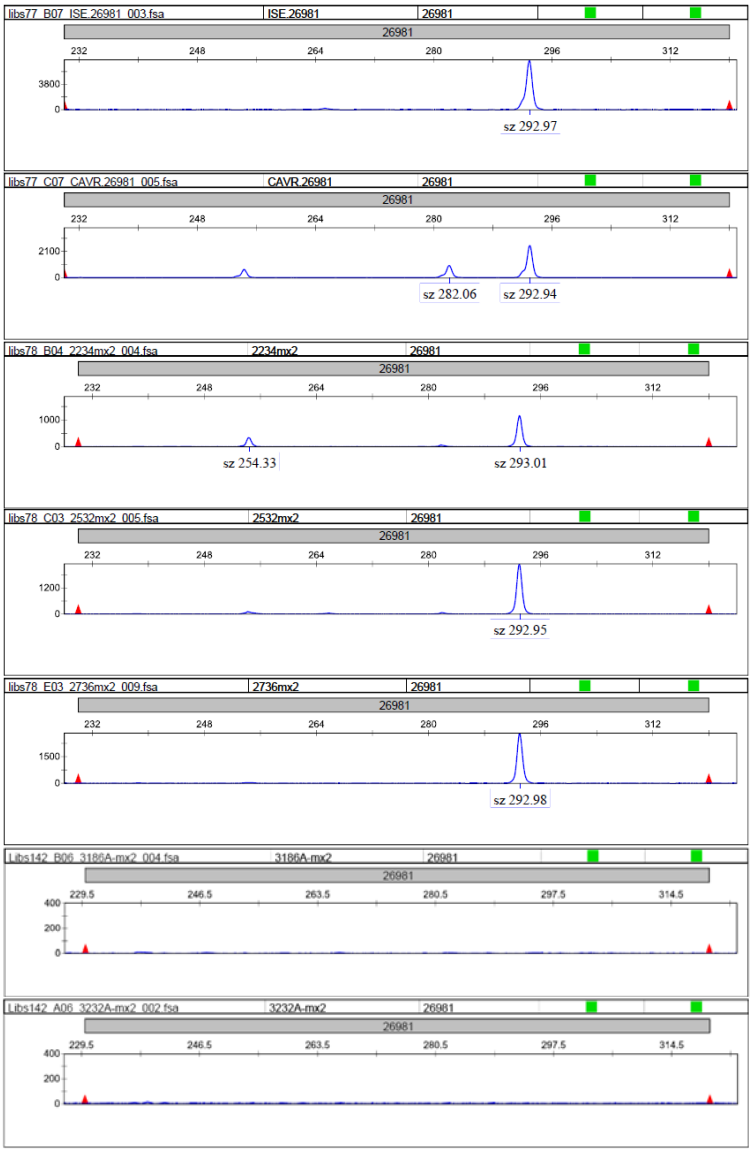
Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **22co3** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



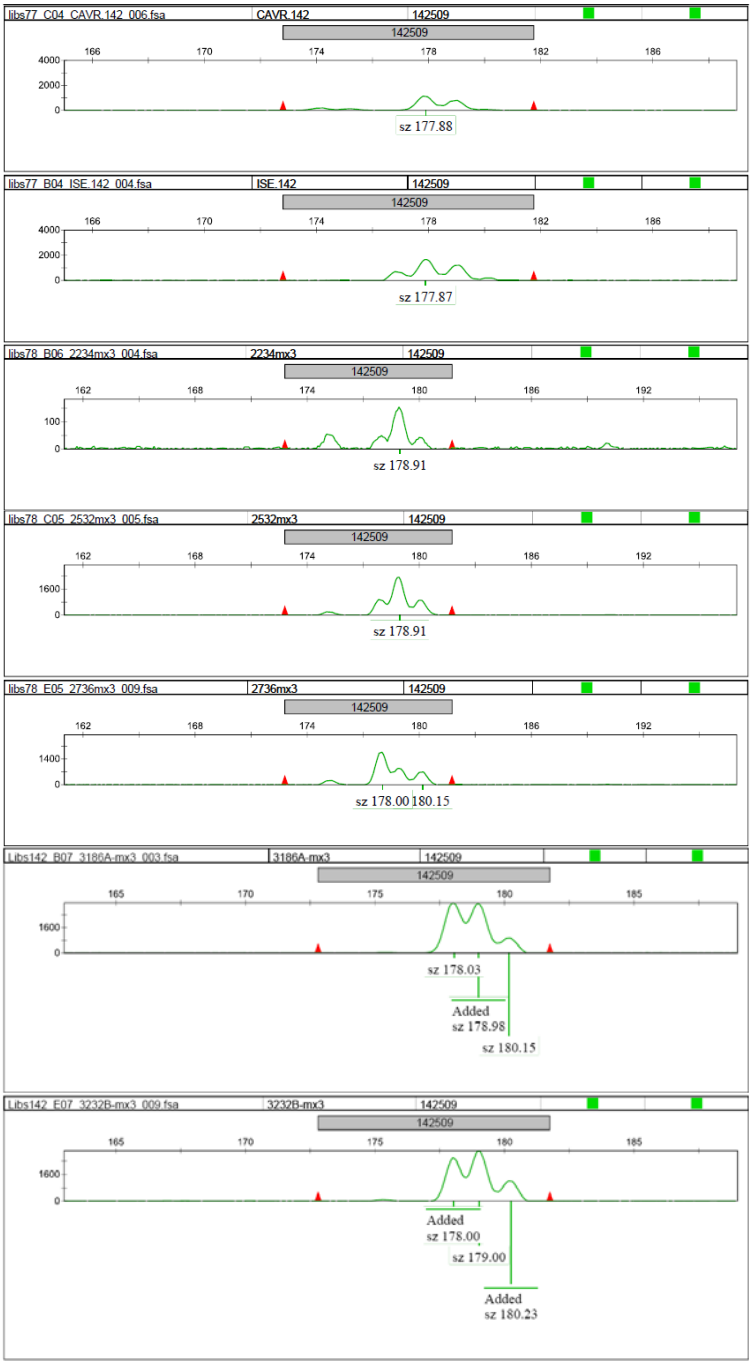
Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **3561** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



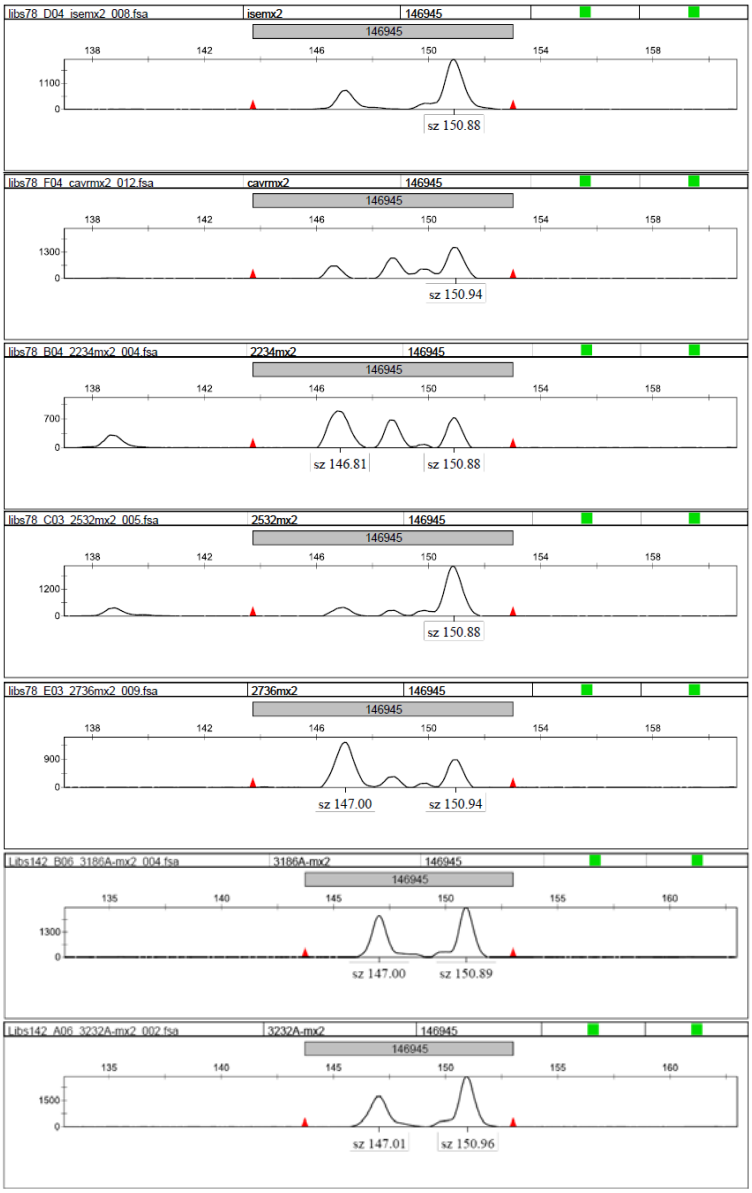
Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **18210** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



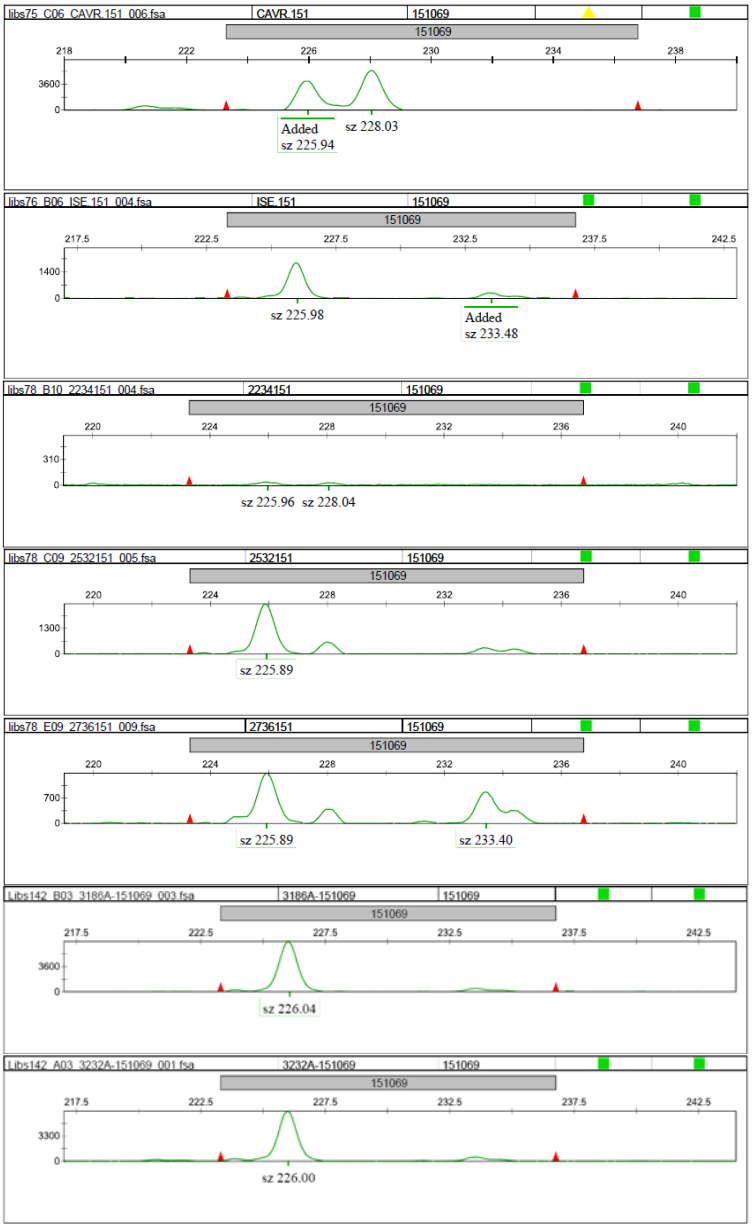
Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **26981** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **X142** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **X146** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco10 (CAVR)

MHco3/10

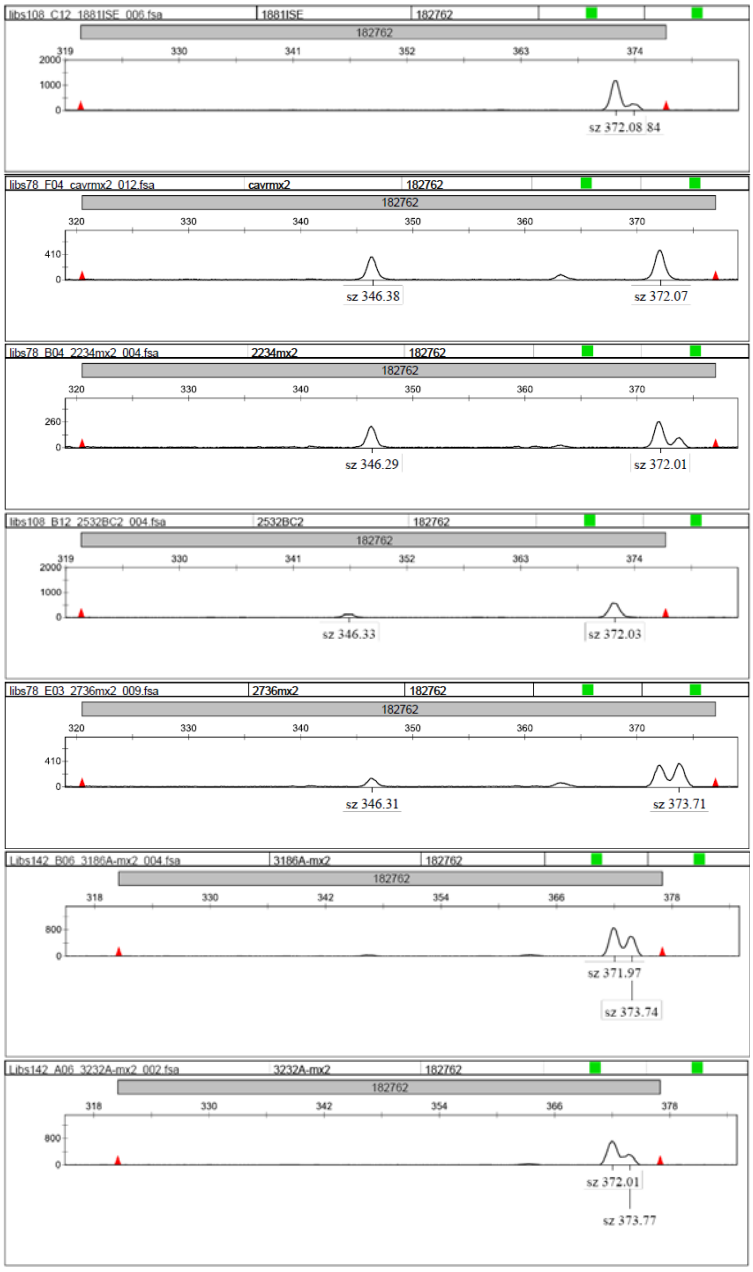
MHco3/10.BC$_1$

MHco3/10.BC$_2$

MHco3/10.BC$_3$

MHco3/10.BC$_4$

Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **X151** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco10 (CAVR)

MHco3/10

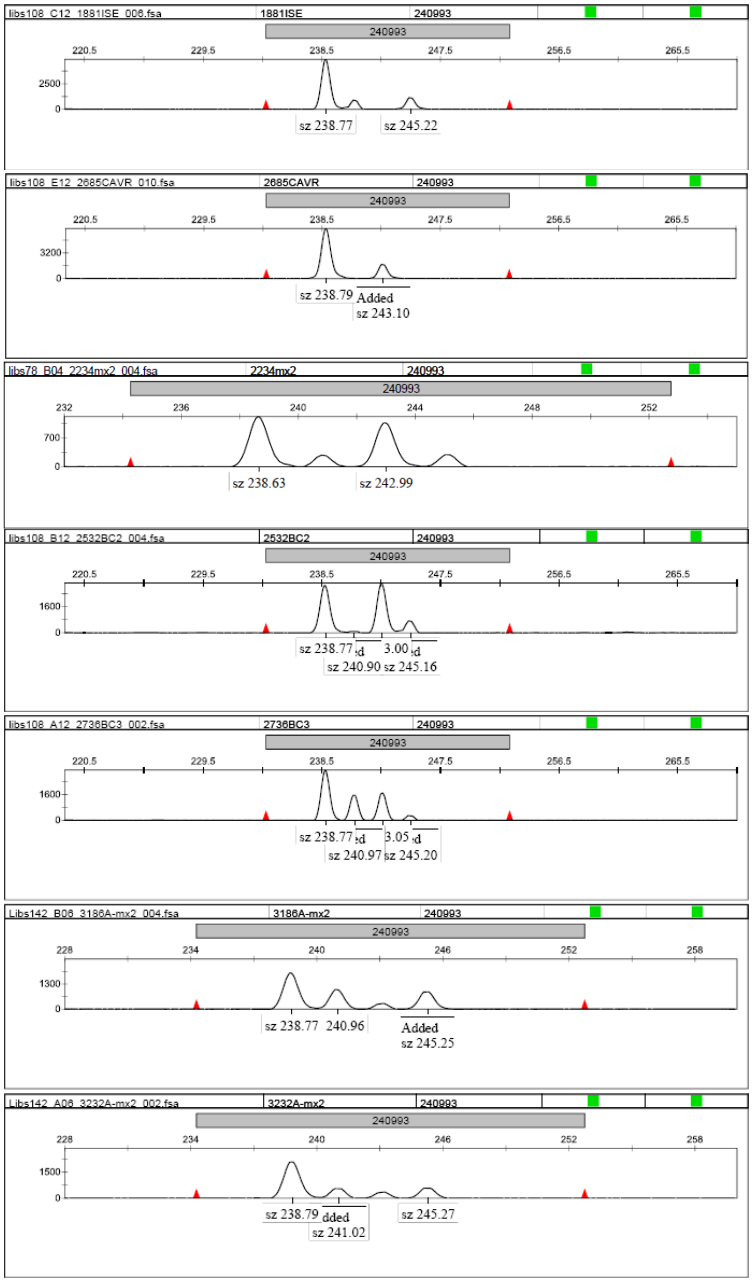
MHco3/10.BC_1

MHco3/10.BC_2

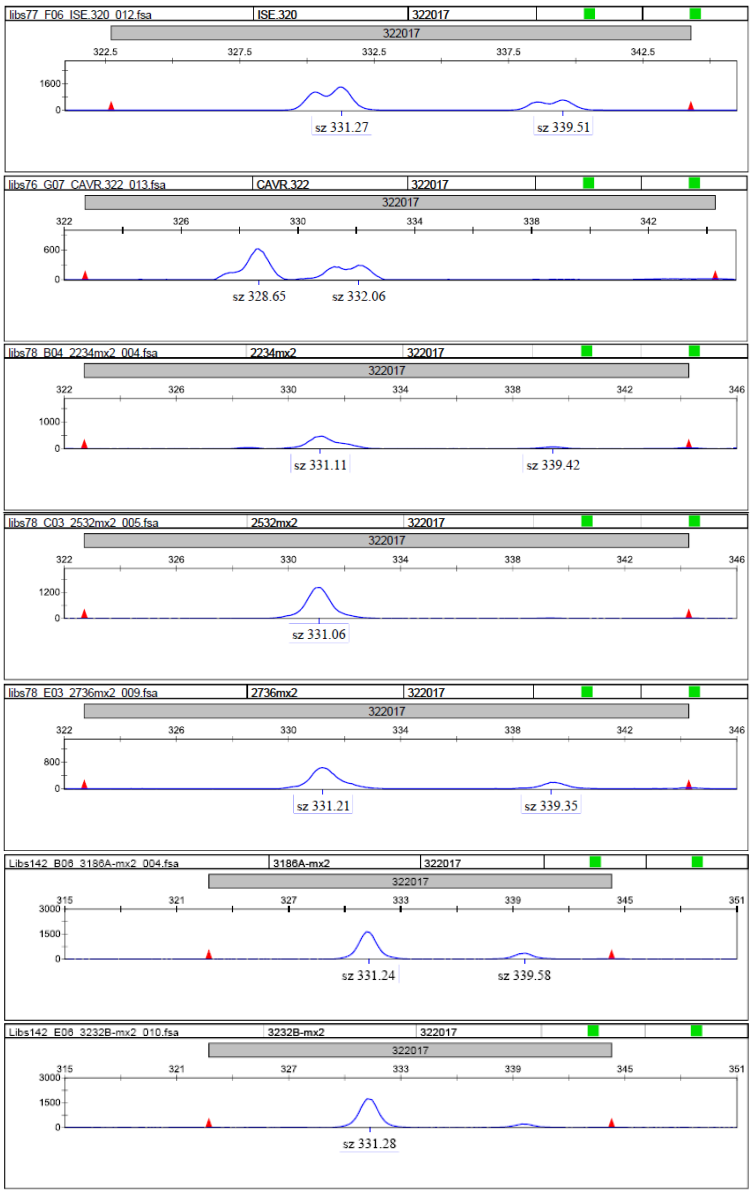
MHco3/10.BC_3

MHco3/10.BC_4

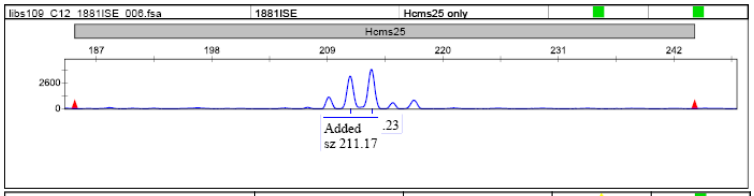
Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **X182** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



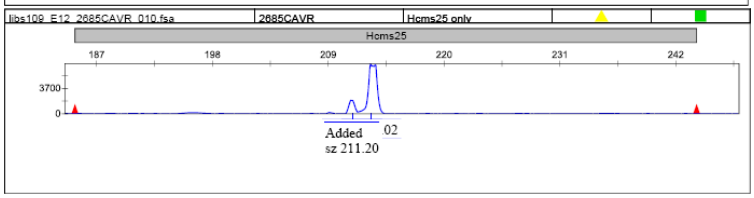
Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **X256** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **X337** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.

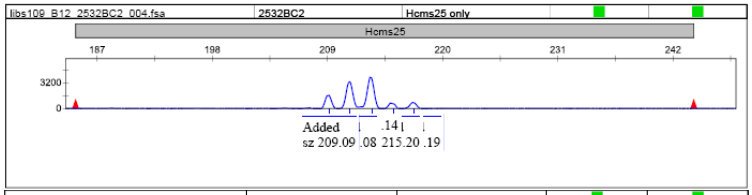


MHco3 (ISE)

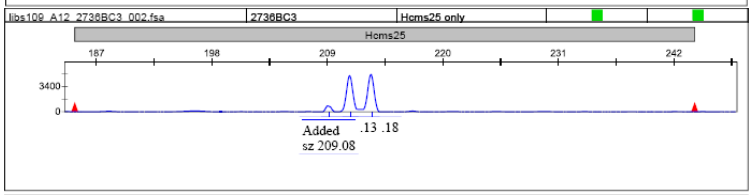


MHco10 (CAVR)

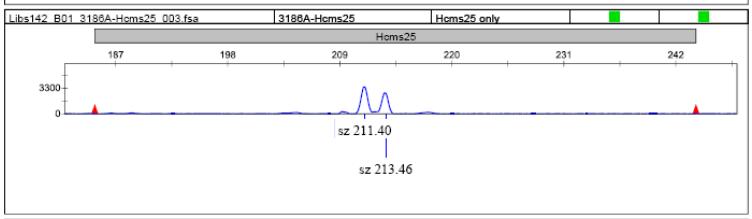
MHco3/10 – no data



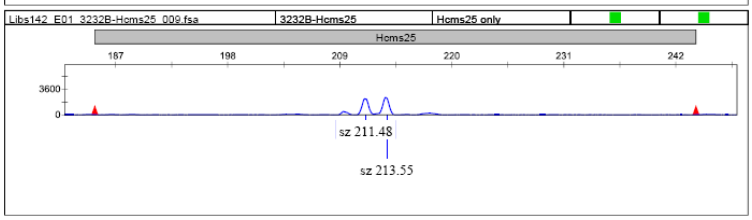
MHco3/10.BC$_1$



MHco3/10.BC$_2$

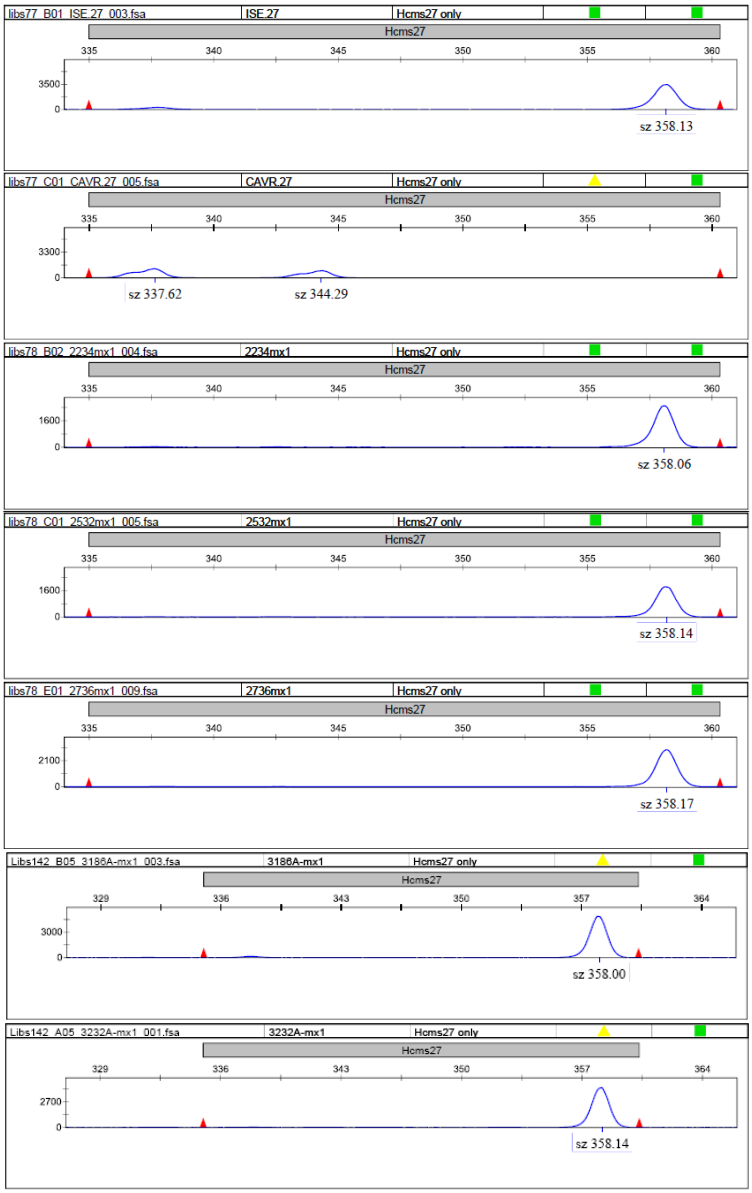


MHco3/10.BC$_3$



MHco3/10.BC$_4$

Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **Hcms25** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco10 (CAVR)

MHco3/10

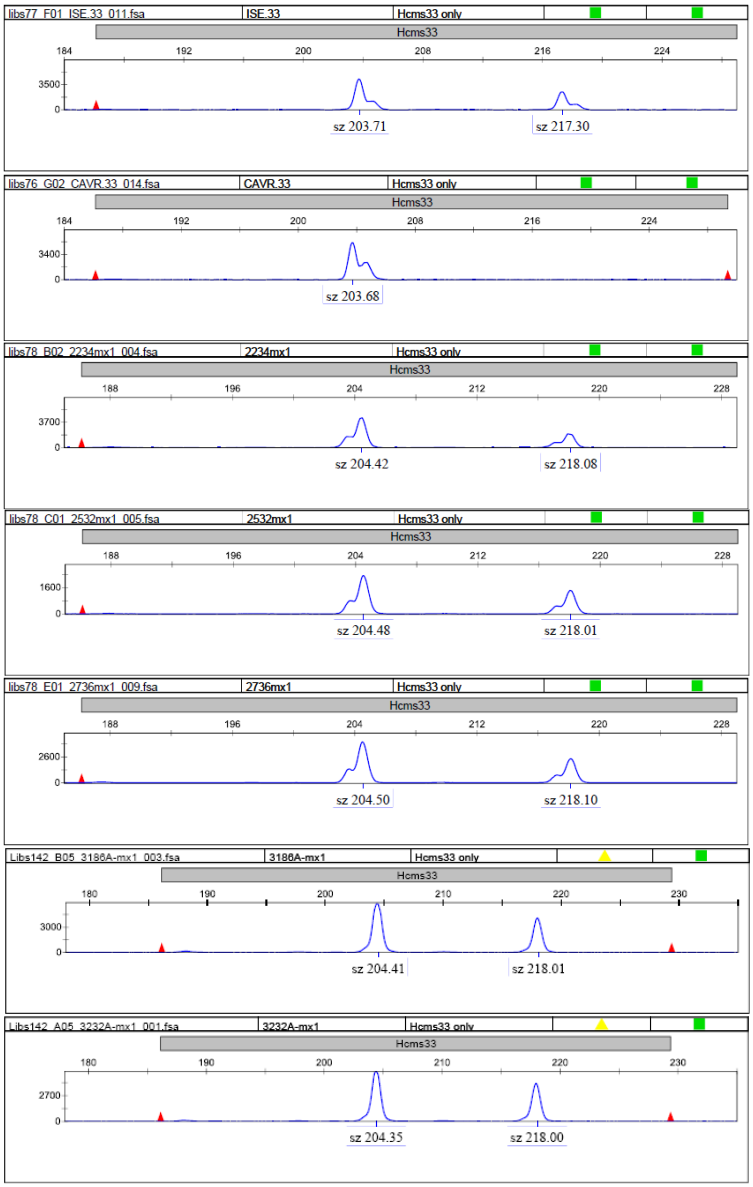
MHco3/10.BC$_1$

MHco3/10.BC$_2$

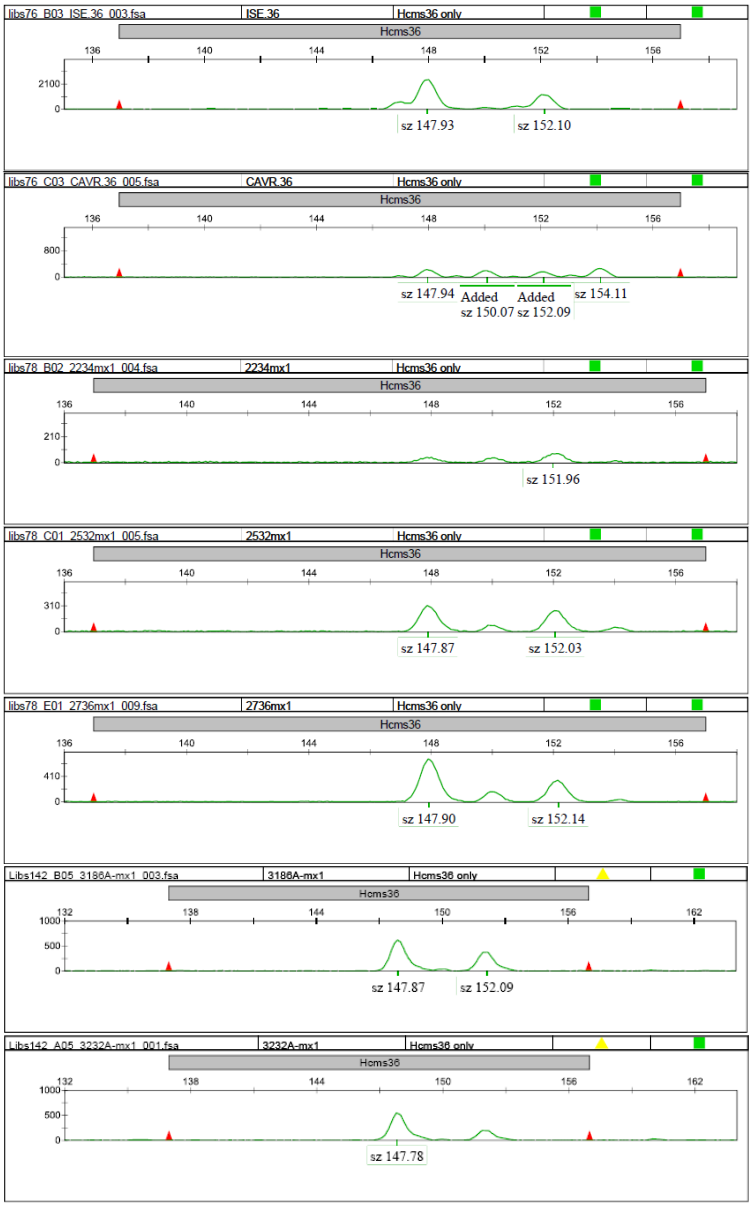
MHco3/10.BC$_3$

MHco3/10.BC$_4$

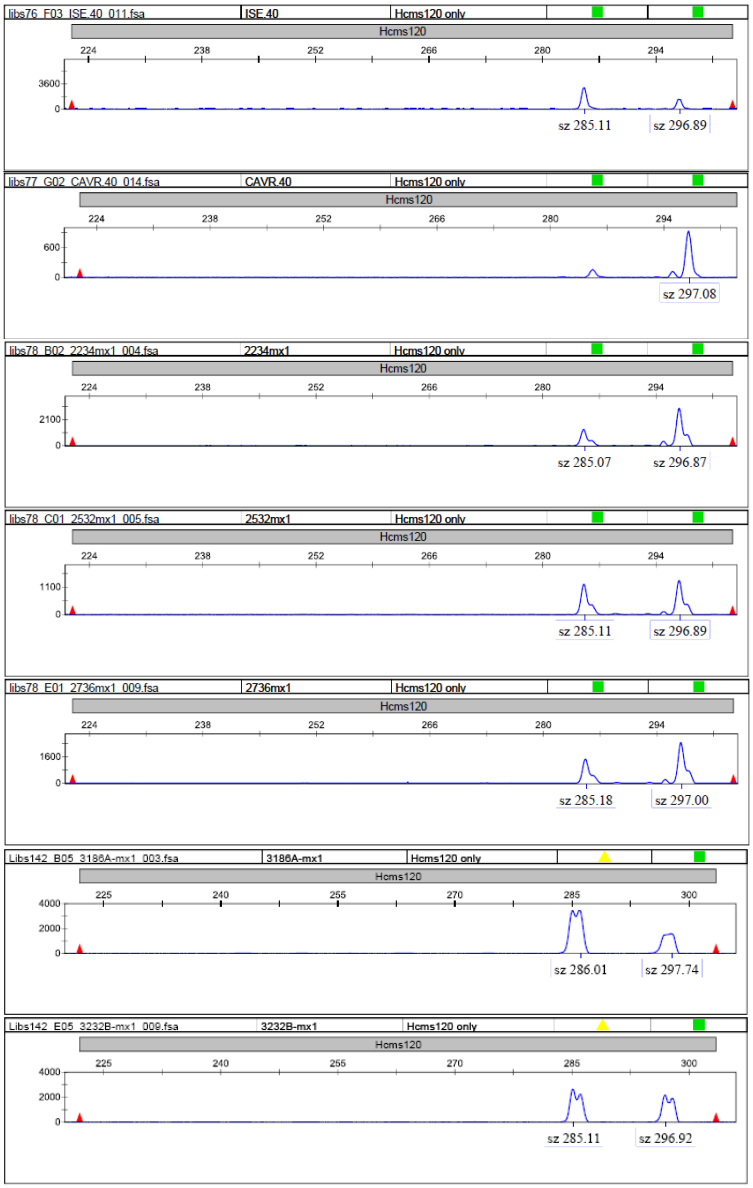
Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **Hcms27** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



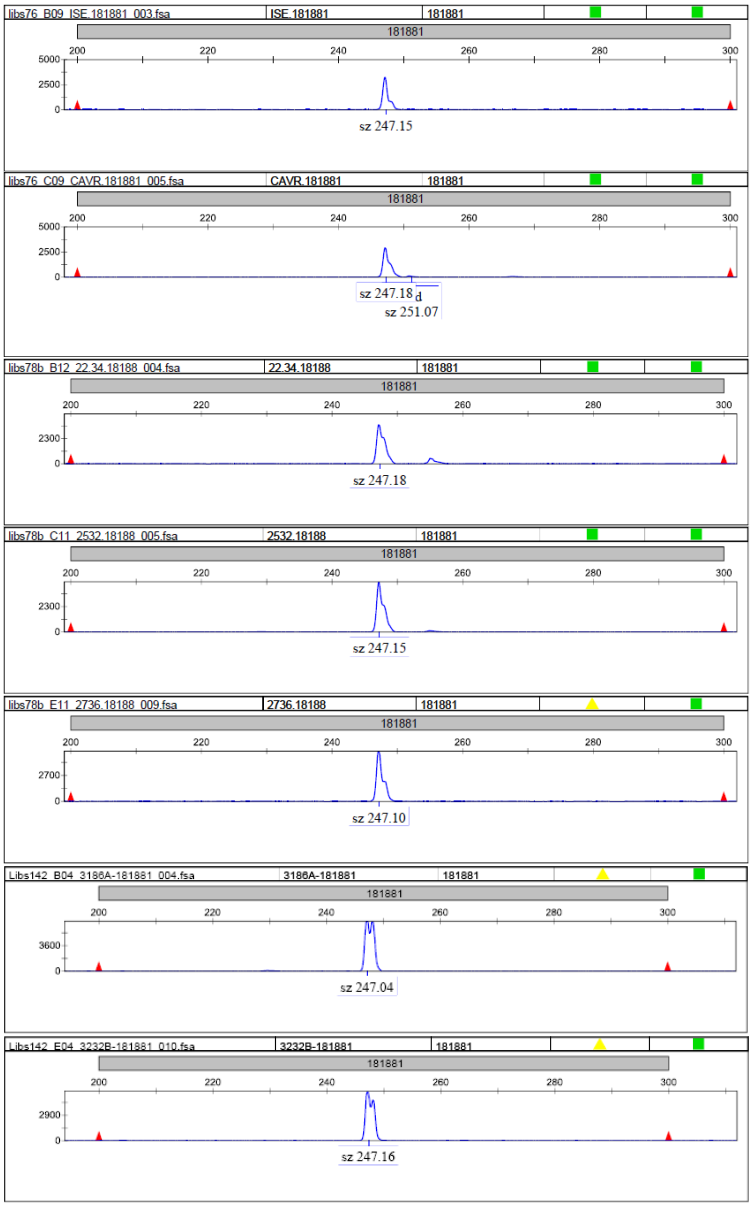
Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **Hcms33** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **Hcms36** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **Hcms40** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.



MHco3 (ISE)

MHco10 (CAVR)

MHco3/10

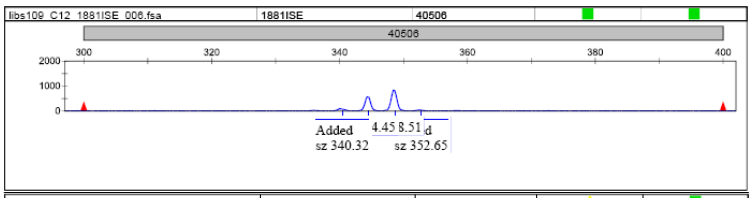
MHco3/10.BC$_1$

MHco3/10.BC$_2$

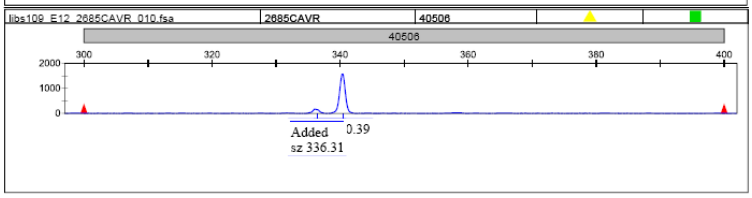
MHco3/10.BC$_3$

MHco3/10.BC$_4$

Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **181881** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.

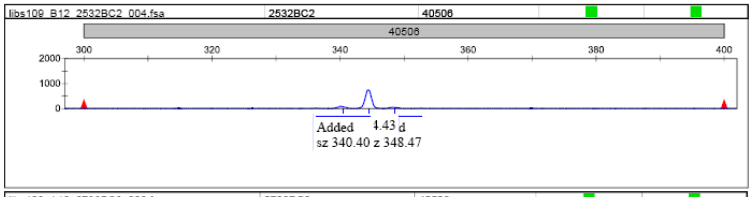


MHco3 (ISE)

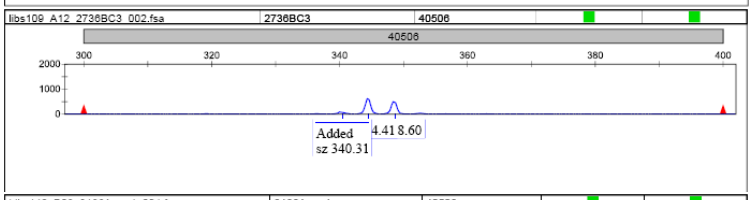


MHco10 (CAVR)

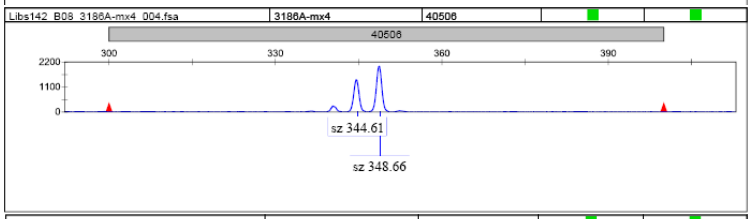
Mhco3/10 no data



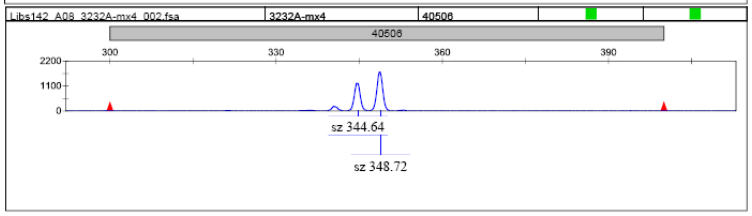
MHco3/10.BC_1



MHco3/10.BC_2



MHco3/10.BC_3



MHco3/10.BC_4

Appendix 5.2: Microsatellite Genescan traces for microsatellite marker **40506** for bulk lysates of the parental MHco3 (ISE) and MHco10 (CAVR) strains of *H. contortus* and for backcross populations. Most of the microsatellite PCRs and Genescan analyses were performed in triplicate. The clearest Genescan traces for each population are shown.